

INVESTIGATIONS ABOUT THE LOCALIZATION
OF THE HEAD ACTIVATOR IN
HYDRA ATTENUATA PALL. (HYDROZOA, CNIDARIA)

INAUGURAL-DISSERTATION

zur Erlangung der Philosophischen Doktorwürde
vorgelegt der
PHILOSOPHISCHEN FAKULTÄT II
der
UNIVERSITÄT ZÜRICH

von
ANDREA MAURER
aus Deutschland

Begutachtet von
Herrn Prof. Dr. P. Tardent

Zürich 1988
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To Hildegard

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EINLEITUNG

Musterbildungsprozesse sind durch Morphogene gesteuerte Wachstums- und Differenzierungsvorgänge. Diese endogenen Wirkstoffe wie der 'Nerve growth factor', der 'Epidermal growth factor' und der 'Fibroblast growth factor' haben hormonellen Charakter und regulieren morphogenetische Prozesse, indem sie eine mitogene Wirkung ausüben und unter anderem epidermale, mesodermale sowie neuronale Zelltypen zu Proliferation und Wachstum anregen (Andres et al., 1977; Cohen and Carpenter, 1975; Gospodarowicz, 1975).

Vergleichbare Morphogene, zu denen nebst Aktivatoren auch antagonistisch wirkende Inhibitoren gezählt werden müssen, sind auch in Entwicklungssystemen von wirbellosen Tieren entdeckt und charakterisiert worden, insbesondere bei Vertretern des Stammes der Cnidaria (Nesseltiere). So haben z.B. Rose und Rose (1941) und Tardent (1960, 1963) beim Hydroidpolypen Tubularia einen Hemmfaktor nachgewiesen, der vom Apicalorgan (Hydranth) des Polypen synthetisiert und damit in seiner Nähe die regenerative Entwicklung eines homologen Organs verhindert.

Später gelang es, bei Süßwasserpolyphen der Gattung Hydra (Hydrozoa, Cnidaria) vier morphogenetische Wachstumsfaktoren nachzuweisen (Schaller et al., 1979; Schmidt et al., 1980). Es handelt sich dabei um den Kopfaktivator und dessen Antagonisten den Kopfinhibitor, welche die Differenzierung der apicalen Organe des Polypen (Hypostom, Tentakel) fördern bzw. hemmen. Ein zweites Antagonistenpaar, der Fussaktivator und der Fussinhibitor steuern die Entwicklung der basalen Teile des Polypen, d.h. des Stiels und der Fussdrüse. Die Verteilung dieser vier Substanzen, die entlang des

Polypen in Form von Gradienten mit einem Maximum im apicalen Teil für die Kopfsubstanzen und einem Maximum im basalen Teil für die Fusssubstanzen vorliegen (Wolpert et al., 1974; Gierer, 1977; Schmidt et al., 1980), und deren Zusammenspiel sind für die Aufrechterhaltung der Polarität des Polypen und im Fall von regenerativen Prozessen für die Musterbildung, d.h. für die Verwirklichung der normalen Gestalt, verantwortlich.

Hydra eignet sich für die Untersuchung derartiger Steuerungsvorgänge aufgrund ihres relativ einfachen Bauplanes, bestehend aus zwei Gewebeschichten, Ekto- und Entoderm, einer überschaubaren Zahl verschiedener Zelltypen (Campbell und Bode, 1983) und ihres aussergewöhnlichen Regenerationsvermögens, wobei Struktur und Gestalt selbst aus einem chaotischen Zellhaufen wiederhergestellt werden können (Gierer et al., 1972). Im Rahmen derartiger Regenerationsvorgänge übernimmt vor allem der apicale Organkomplex, über den Kopfkaktivator, die Rolle eines Organisators.

Mit der Isolation dieses Kopfkaktivators (Schaller, 1973) wurde eine Substanz in Hydra gefunden, die die im Modell von Gierer und Meinhardt (1972, 1974) postulierten Eigenschaften eines wachstumsfördernden Morphogens aufweist, indem sie die Neubildung des Kopfes sowie die Entstehung vegetativ gezeugter Knospen (asexuelle Vermehrung) induziert; ferner als Wachstumshormon wirkt, indem Zellen, die in der G_2 -Phase des Zellzyklus blockiert sind, stimuliert werden eine Mitose zu durchlaufen, was zu einer allgemeinen Erhöhung des mitotischen Indexes führt (Schaller, 1976a). Ferner wird die Differenzierung von undifferenzierten, multipotenten interstitiellen Stammzellen (I-Zellen) zu Nervenzellen induziert (Schaller, 1976b; Holstein et al., 1986).

Der Kopfkaktivator, der mittlerweile in chemisch reiner Form (HPLC) isoliert und sequenziert werden konnte (Schaller und Bodenmüller, 1981; Birr et

al., 1981), ist ein saures Peptid bestehend aus 11 Aminosäuren (pGlu-Pro-Pro-Gly-Gly-Ser-Lys-Val-Ile-Leu-Phe) mit einem Molekulargewicht von ca 1300 d, das in sehr geringen Konzentrationen (10^{-16} M/Hydra) vorkommt.

Das Peptid dimerisiert sehr leicht, ist jedoch nur in der monomeren Form in Konzentrationen unter 10^{-13} M aktiv. Um diese Form zu erhalten, wird der Kopfaktivator zusammen mit einem Carrier-Molekül sezerniert (Schaller et al., 1986). Die Struktur dieses Peptids scheint phylogenetisch streng konserviert zu sein, denn ein Peptid mit der gleichen Sequenz (Bodenmüller und Schaller, 1981) wurde unter anderem in embryonalen Gehirnen von Ratten und anderen Säugern, bei Drosophila, Fröschen, in menschlichem Hypothalamus sowie im Serum und in Tumorzellen neuronalen und endokrinen Ursprungs nachgewiesen (Schaller et al., 1977; Bodenmüller et al., 1980).

Der Kopfaktivator soll bei Hydra zu 90-95% strukturgebunden und zu 5-10% in freier Form vorliegen. 70% der Aktivität wurden in Membranstrukturen nachgewiesen, deren Dichteigenschaften auf das endoplasmatische Reticulum hinweisen. 80-90% der Aktivität entfallen auf die Nervenzellen (Schaller und Gierer, 1973; Grimmelikhuijzen und Schaller, 1979).

Es ist möglich, nervenzellfreie Hydren experimentell herzustellen, indem durch geeignete Behandlung mit Colchizin oder Hydroxyharnstoff die I-Zellen selektiv entfernt werden. Zusammen mit den I-Zellen verschwinden dann auch deren Differenzierungsprodukte (Nervenzellen, Drüsenzellen und Nematocyten). Wie Marcum and Campbell (1978) zeigen konnten, wachsen und regenerieren solche aus rein epithelialen Zellen bestehende Polypen normal, und die Axial-Polarität bleibt auch bei völliger Abwesenheit der oben erwähnten Zelltypen gewährleistet. Diese Befunde stellen die Rolle des Nervensystems in Zusammenhang mit der Steuerung morphogenetischer Prozesse bei Hydra einerseits und die Nervenzellen als ausschliessliche Quelle des Kopfaktivators andererseits in Frage. Es hat sich sogar



herausgestellt, dass epitheliale Hydren 8x mehr Kopfaktivator enthalten als normale Tiere (Schaller et al., 1980a). Dies lässt vermuten, dass ausser den Nervenzellen noch andere Zelltypen in der Lage sind, den Kopfaktivator zu produzieren. Gemäss Schaller et al. (1980b) verfügen auch Epithelzellen über die Fähigkeit, den Aktivator zu produzieren. Diese Fähigkeit würde nach Auffassung der Autoren, im Normalfall, d.h. unter Anwesenheit der Nervenzellen, unterdrückt. Mittels Dichtegradientenzentrifugation in Percoll konnten Epithelzellen von normalen Hydren angereichert und ein Aktivatorgehalt von ca 9% nachgewiesen werden. Diese Präparate enthielten jedoch Epithelzellen sowohl der ektodermalen, als auch der entodermalen Zellschicht, sodass nicht feststeht, welchem der beiden Zelltypen die registrierte Aktivität zuzuordnen ist und ob noch andere Zelltypen Kopfaktivator produzieren oder speichern können. Versuche, den Aktivator immunocytochemisch in einem bestimmten Zelltyp zu lokalisieren, scheiterten daran, dass er nicht mit den üblichen Methoden fixiert werden kann (Bodenmüller und Roberge, 1985).

Die Frage nach dem Produktions- bzw. Speicherort des Kopfaktivators von Hydra ist auf dem Stand der Kenntnisse nach wie vor noch kontrovers. Die vorliegende Arbeit hatte es sich zum Ziel gesetzt, einen alternativen Weg zu finden, um den Kopfaktivator in den verschiedenen Zelltypen von Hydra und in subzellulären Fraktionen nachzuweisen.

Das Konzept des Projektes umfasste die folgenden drei Stufen: Lokalisation des Kopfaktivators 1.) in den beiden Epithelien (Ekto- und Entoderm), 2.) in bestimmten Zelltypen beider Schichten und 3.) in subzellulären Fraktionen.

Für den ersten Schritt wurde die Methode von Epp et al. (1979) angewendet, nach der die beiden Gewebeschichten in lebendem Zustand und relativ sauber voneinander getrennt werden können. Diese Isolate wurden in Form von Roh- und Methanolextrakten auf ihre Aktivatoraktivität hin

untersucht. Versuche, die Anwesenheit des Aktivators flankierend mit Hilfe der Implantationsmethode (Sugiyama, 1982) nachzuweisen, erwiesen sich im Fall der ektodermalen Implantationen als erfolgreich, scheiterten aber an der Tatsache, dass die wesentlich fragileren Isolate des Entoderms während oder kurz nach deren Implantation in den Wirtspolypen zerfielen.

Der zweite Schritt erforderte zunächst die Entwicklung neuer Methoden zur Isolation einzelner Zelltypen von Hydra. Die dafür erforderliche Dissoziation in Suspensionen von lebenden Einzelzellen bedurfte ebenfalls der Entwicklung eines neuen Procedere, da die geläufigen Dissoziationsverfahren eine geringere Ausbeute an einzelnen Zellen lieferten und ferner einige der als Oberflächenmarker verwendeten Lektine herkömmlich dissoziierte Zellen nicht erkannten.

Zur selektiven Markierung der verschiedenen Zelltypen wurden FITC-conjugierte Lektine, das Schlangengift α -Bungarotoxin und ein monoklonaler Antikörper verwendet. Das Isolationsverfahren umfasste eine Anreicherung der gewünschten Zelltypen mittels Percoll-Dichtegradientenzentrifugation. Die dadurch gewonnenen Fraktionen wurden anschliessend durchflusszytometrisch zu einem Reinheitsgrad von mindestens 90% getrennt, methanolextrahiert und auf ihren Gehalt an Kopfaktivator hin analysiert.

Die in der vorliegenden Arbeit neu entwickelte Methode zur Trennung bestimmter Zelltypen von Hydra erfordert in Einzelfällen noch eine Verfeinerung. So wäre z.B. eine saubere Trennung von interstitiellen Zellen und frühen Stadien von Nematoblasten erstrebenswert. Das Trennungsverfahren eröffnet eine Reihe neuer Möglichkeiten, unter anderem die Voraussetzungen für die Herstellung von spezifischen, monoklonalen Antikörpern gegen die einzelnen Differenzierungsstadien eines Zelltypes, ausgehend von monotypischen Isolaten.

Um den Kopfaktivator auch intrazellulär lokalisieren zu können, wurden in einem dritten Schritt Zytosol- und Membranfraktionen aus Homogenaten ganzer Hydren isoliert. Der Reinheitsgrad der Fraktionen wurde mit den entsprechenden typischen Leitenzymen untersucht und Zytosol sowie Membranen methanolextrahiert und auf ihre Aktivatoraktivität hin untersucht.

Als Assay-System für den Nachweis der Aktivatoraktivität wurde die von Holstein et al. (1986) entwickelte Methode verwendet, die der bereits erwähnten Wirkungsweise des Kopfaktivators, die Differenzierung von I-Zellen zu Nervenzellen zu induzieren, zugrunde liegt. Dieses Testsystem erwies sich als wesentlich sensitiver als die in den bisher zitierten Arbeiten benützten Systeme.

Die Ergebnisse dieser Untersuchungen sind in den drei folgenden publikationsbereiten Manuskripten dargestellt, von denen sich die ersten zwei mit der Isolation bestimmter Zelltypen auseinandersetzen, während die dritte Arbeit der Lokalisation des Kopfaktivators gewidmet ist.

ISOLATION OF INTERSTITIAL STEM CELLS OF HYDRA (HYDROZOA, CNIDARIA) BY FLOW SORTING

ABSTRACT

Live interstitial stem cells (I-cells) and nematoblasts of Hydra have been isolated by fluorescence activated cell sorting (FACS). With the lectin from Phytolacca americana (Pokeweed) which specifically binds to the mentioned cell types it was possible to isolate large and pure yields of viable cells for biochemical and molecular analysis. Cells were sorted to > 96% purity and 99% viability as demonstrated by propidium jodid exclusion. A new method for the dissociation of Hydra polyps into single cells by means of a temperature- and collagenase treatment was found to facilitate the specific binding of the lectin.

INTRODUCTION

Stem cells represent a cell type of particular interest both in mammals and invertebrates, because throughout the life span of an individual they retain genotypic pluri- or totipotency and the capacity to proliferate. By phenotypic differentiation they replace damaged functional cells.

Due to its simple architecture based on a limited number of cell types (Campbell and Bode, 1983) and to its somatic immortality (Brien, 1953), Hydra, a small polypoid freshwater coelenterate, provides an excellent system for the investigation of stem cell proliferation and differentiation in a multicellular organism as well as the mechanisms controlling these processes.

The tubular body column of a mature animal is composed of about 80'000 cells representing the following three autoreproductive cell lineages, each consisting of a stem cell population and several differentiation products: 1. ectodermal epithelio-muscular cells; 2. endodermal epithelial (digestive) cells and 3. interstitial cells (I-cells). The latter constitute a highly mobile (Tardent and Morgenthaler, 1966) and rapidly proliferating multipotent stem cell population located within the intercellular spaces of the ectodermal epithelium. These stem cells replace the four types of nematocytes (Lehn, 1952; Slautterback and Fawcett, 1959), nerve-and sensory cells (Davis, 1974), endodermal gland cells (Bode et al., 1987) as well as gametes (Brien, 1961) whenever the polyp is engaged in sexual activities (Tardent, 1966).

A biochemical and molecular analysis of particular cell types requires the isolation of individual cells of a specific type. So far, interstitial cells of Hydra could be enriched by flow sorting due to the different light scattering properties of dissociated cells from normal and I-cell free Hydra (Yaross, 1986).

The present paper describes a method for the isolation of large and pure populations of viable interstitial cells and early stages of nematoblasts by FITC-lectin directed flow sorting (Herzenberg and Herzenberg, 1978). The critical requirement for this method was the availability of a suitable surface marker for the cell type in question. Plant lectins can be specific probes for the identification and separation of particular cell types within complex cell populations. With a high degree of specificity they interact with mono- and oligosaccharides present on cell surfaces, thus revealing populations and subpopulations of cells featuring minor differences in surface carbohydrates (Lis and Sharon, 1973). We were able to show that the lectin from Phytolacca americana (Pokeweed) specifically labels small and big interstitial cells, nematoblasts as well as mature nematocytes.

Various methods for dissociating Hydra polyps into either prefixed single cells (maceration method: David, 1973) or into suspensions of live cells (dissociation methods: Gierer, 1974; Flick and Bode, 1983) have been described; however, the above mentioned lectin did not bind to cells subjected to these procedures. We therefore developed a new, more suitable method for the dissociation of Hydra polyps by combining a temperature (Schmid et al., 1981) with a collagenase treatment. This procedure appears to unmask binding sites on the cell surface, thus, enabling the cell type specific lectin binding.

Another important prerequisite for flow sorting of large numbers of cells is the maintenance of a suspension of single cells over a prolonged period of time. Since, dissociated cells of Hydra tend to form clumps in suspensions of low density, an important step in sample preparation was the enrichment of the cells in question on a Percoll density gradient. The subsequent sorting while they were still suspended in this dense medium prevented clumping. Due to their high density (Weber et al., 1987) the lectin-labeled mature nematocytes could easily be separated from I-cells and early nematoblasts by centrifugation prior to flow sorting.

MATERIAL AND METHODS

Culture Conditions

Polyps of Hydra attenuata Pall. were mass cultured in a medium (Loomis and Lenhoff, 1956) at 14°C in plastic trays. They were fed daily with freshly hatched larvae of Artemia. Animals used for the experiments were collected from the cultures not before 24 hr after the last feeding.

Dissociation Procedure

For each experiment approximately 200 polyps were dissociated into single cells in 900 μ l of a medium containing dissociation buffer (5 mM CaCl_2 , 2 mM MgSO_4 , 54 mM KCl, 20 mM Tris, 6 mM Na-Pyruvate, 6 mM Na_2 -Citrate, adjusted to pH 7.5) and Collagenase from Clostridium histolyticum (Serva Heidelberg; 0.74 U/mg, 5 mg/ml dissociation buffer) in a ratio of 8:1.

The dissociation medium was preincubated at 37°C for 30 min to activate the collagenase. Polyps were collected from the mass culture, washed three times in culture medium, concentrated by centrifugation (200 x g) and transferred to the prewarmed medium. After 10 min of incubation the animals were resuspended once with a Pasteur pipet. Following an additional 20 min of incubation, dissociation was achieved by gentle mechanical disruption by two complete passes through a Pipetman P-1000 pipettor fitted with a blue tip. Incompletely dissociated cell clumps were allowed to gravity sediment for 20 s. The suspended single cells were processed further immediately. Propidium jodid (Fluka, Buchs, Switzerland) staining was used for monitoring the viability of the cells. Cell counts were made with a hemacytometer.

Lectin Staining

Fluorescein-conjugated lectin from Phytolacca americana (Pokeweed) was obtained from Sigma. Approximately 1.8 ml of the cell suspension containing about 1.6×10^7 cells were added to 1.2 ml of lectin (300 $\mu\text{g/ml}$ dissociation buffer) in a 15 ml Corning tube and incubated under constant gentle rotation for 10 min at room temperature. The labeled cell suspension was immediately transferred to the Percoll gradient. As a control for autofluorescence a batch of cells was always incubated in 3 ml of dissociation buffer lacking the FITC-conjugated lectin. The specificity of the lectin binding was demonstrated with N-acetyl-glucosamine which at a concentration of 100 mg/ml prevents the binding of the lectin.

Percoll Gradient

During lectin incubation a fraction of the cells tended to clump. These cell masses were allowed to gravity sediment for 20 s. Approximately 3 ml of the supernatant containing lectin-labeled single cells only were layered onto a three-step Percoll density gradient (Pharmacia, Uppsala, Sweden; 30%, 60 %, 80% (v/v), 3 ml per step) in a 10 ml centrifuge tube. Care was taken that both the cell suspension and the gradient had the same temperature. The gradient was centrifuged at $2'000 \times g$ for 2 hr in a Sorvall centrifuge (RT 6'000; H-1000B, swing-out rotor). The cells which had accumulated at the two interfaces were collected from the top with a Pasteur pipet. The density of the interbands was determined by means of density marker beads (Pharmacia, Uppsala, Sweden).

Flow Cytometry

A Cytofluorograf 50H (Ortho Diagnostic Systems, Inc., Westwood, MA), interfaced to a 2151 computer (Ortho Diagnostic Systems) was used for the analysis and the sorting of individual cell types. An argon-ion laser (Spectra Physics 164-05) operating at 488 nm and 100 mW was used to excite FITC.

Forward small-angle light scatter and 90° scatter together with green fluorescence of 20'000 cells were analyzed. Fluorescence was measured on a 3.5 decade logarithmic scale and was separated from the 90° scattered light by a 500 nm long pass dichroic mirror and a 510 to 550 nm band pass filter. The scatter signals were both measured in linear mode. Sorting was maximized for optimal recovery and 3 droplets per cell were deflected. Cells were sorted with a droplet generation frequency of 32 kHz at a flow rate of 1000 cells/s through a 75 µm orifice and collected in an Eppendorf tube filled with 500 µl dissociation buffer. Approximately 150'000 cells were sorted per Eppendorf tube.

Sorting efficiency was determined by sorting about 200 cells onto a microscope slide and counting the relative number of fluorescent cells.

Whole Mounts

Whole polyps of Hydra were mounted according to a slightly modified procedure outlined by Littlefield et al. (1985). Intact animals were allowed to relax for 30 s in 2% urethane. They were subsequently fixed in Lavdowsky fixative for 30 min and then rinsed with 70% EtOH, washed in PBS, placed in wells of a 24-well plate (Costar) and incubated in PBS-NCS for 2 hr. After a short rinse with PBS, the animals were washed in PBS for 30 min and

incubated in FITC-labeled Phytolacca americana for 1 hr. Afterwards they were again washed in PBS for 30 min and mounted on microscope slides in a medium containing 70% glycerol, 5% N-propylgallate in 0.1 M Tris, buffered at pH 9.2 to reduce photobleaching (Giloh and Sedat, 1982). Whole mounts of polyps stained with toluidine blue were prepared according to the procedures described by Diehl and Burnett (1964) and Littlefield (1985). Animals were fixed for 1-2 min in absolute alcohol, washed twice in distilled water and stained for 45 s in 0.05% toluidine blue buffered in Tris at pH 7.4. Afterwards the polyps were dehydrated through an ethanol and xylol series and mounted in Euparal (Chroma, Köngen, BRD).

Microscopy

FITC and propidium iodid-labeled cells as well as whole mounts were analyzed by using a Zeiss Fluorescence Microscope (Photomikroskop II, Zeiss, Oberkochen, FRG) equipped with phase contrast optics, a Reichert Jung Fluorescence Microscope (Polyvar, Vienna, Austria) and a Zeiss Axiophot, both provided with Nomarski illumination.

RESULTS

The cell type specificity of the Phytolacca lectin binding was visualized in whole mounts of Hydra polyps. Animals which had been incubated with FITC-conjugated lectin contained both fluorescent small and big interstitial cells (I-cells), nests of fluorescent nematoblasts (Fig. 1a) and mature nematocytes. It had been shown previously (Tardent, 1954; Diehl and Burnett, 1964; Heimfield and Bode, 1984) that in whole mounts toluidine blue

stains I-cells more intensely than the surrounding epithelial tissue in which they are imbedded (Fig. 1b). Our comparison between FITC-lectin treated whole mounts and toluidine blue stained homologues revealed that in both cases, the same types of cells (I-cell) were labeled.

Hydra which had been treated with hydroxyurea gradually lose their I-cell population and consequently all differentiation products of these stem cells, namely nerve- and sensory cells, nematocytes and gland cells (Diehl and Burnett, 1964; Littlefield, 1985). Such epithelial polyps were dissociated and exposed to the lectin. In these preparations an extremely small amount of labeled cells with the typical morphological feature of I-cells (Fig. 2), probably relicts of not completely exterminated I-cell populations, were found. This finding additionally demonstrates that the cells in question to which Phytolacca americana binds specifically are I-cells.

As a control for possible nonspecific adsorption of the lectin we co-incubated dissociated cells with Phytolacca and a high concentration of N-acetyl-glucosamine, a competing sugar. In this case the lectin had bound to the sugar in solution and no labeling of cells was detected.

The new dissociation method allows an almost complete disintegration of the polyps into single cells including their tentacles which are somehow refractory to other dissociation procedures (Gierer, 1974; Flick and Bode, 1983). However, for yet unknown reasons, attempts to reaggregate the suspended cells following the heat-collagenase treatment in order to obtain morphogenetically active regenerates failed.

In media with low densities dissociated cells of Hydra tend to form clumps. Yet, suspensions of single cells are required for fluorescence activated cell sorting. To overcome these clumping tendencies we enriched the cells on a 3-step discontinuous Percoll density gradient (3-fold enrichment) and

successfully sorted the cells while they were still suspended in this dense medium. Each sample layered on top of a gradient contained approximately 1.6×10^7 cells. After centrifugation unwanted cellular debris of low density and damaged cells to which lectins tend to bind unspecifically (Pertoft and Laurent, 1977) could be removed from the top of the tube and were discarded. The living cells were recovered mainly at the two interfaces between the Percoll cushions. Each of these two fractions contained cell populations featuring partly overlapping size and density properties. As shown in table 1 the first interface (density between 1.049-1.075 g/ml) included epithelial cells of the ecto- and endoderm and cells of other types. The second interface with a density ranging between 1.087 and 1.098 g/ml was predominantly composed of I-cells, nerve- and sensory cells, endodermal gland cells as well as desmoneme nematocytes and few other cell types (table 2). The majority of the mature nematocytes (stenoteles, desmonemes and isorhizas) could be recovered from the bottom of the tube (table 3). The viability of the separated cells was checked by means of propidium jodid exclusion (Dangl et al., 1982).

Small and big I-cells as well as nematoblasts in the second Percoll interface are not distinguishable from each other on morphological criteria. The analysis of the fluorescent cell population by the FACS could not reveal distinct subpopulations. The sorted population of cells therefore was a mixture of these differentiation stages of I-cells. Fluorescent nematocytes could be mostly eliminated from the population of cells to be sorted by centrifugation in the Percoll gradient and only few cells of this type were found in the second interface.

The cell suspensions were analyzed in cytograms correlating forward scatter vs 90° scatter. The profile of the FITC-labeled cell population is shown in Fig. 3. An analysis window was defined around the zone containing

dense populations of cells (region 1). Fig. 4 and 5 show the analysis in ungated cytograms correlating forward scatter vs logarithmic fluorescence and a histogram of fluorescence gated on the region containing the population of interest in the scatter cytogram. The control exhibited only minor autofluorescence, whereas approximately 70-80% of the total lectin-treated cell populations were positively labeled l-cells and nematoblasts. A sorting window around the FITC-positive population was defined in region 2. Cells were sorted to 96% purity and 99% viability.

DISCUSSION

The present study demonstrates that relatively large and pure populations of viable interstitial stem cells (l-cells) and early stages of nematoblasts can be jointly isolated from heterogeneous cell suspensions of Hydra using lectin-directed flow sorting. The fact that more than 40'000 cells per min and more than 2 million cells per experimental run can be isolated proves the usefulness of this technique in providing sufficient amounts of material required for a biochemical and molecular analysis of these particular cell types.

This isolation procedure became possible due to the discovery that the cell surfaces of l-cells specifically interact with the lectin from Phytolacca americana. However, this lectin binding does not occur when the dissociation of the polyps is performed according to current methods (Gierer, 1974; Flick and Bode, 1983). The heat dissociation (Schmid et al., 1981) combined with the collagenase treatment, revealed to be more successful. We assume, that, as found with neuraminidase treatment (Notter and Leary, 1987), the adopted procedure unmaskes the recognition sites which interact specifically with the lectin in question.

The latter binds not only to uncommitted l-cells but also to the surface of both nematoblasts (precursors of nematocytes) and mature nematocytes of all four types (stenoteles, desmonemes, holotrichous and atrichous isorhizas). Due to their exceptionally high density (1.22-1.24; Weber et al., 1987) the mature nematocytes featuring fully differentiated cysts could be easily removed by density gradient centrifugation prior to flow sorting. The remaining fraction of lectin-labeled cells, therefore, contain uncommitted big and small l-cells as well as early stages of nematoblasts.

Fully differentiated nerve cells show no interaction with the lectin. Whether nerve precursors (early stages of neuroblasts) are also labeled could not be ascertained. Unlike in preparations obtained by maceration of polyps into single prefixed cells (David, 1973) where the original morphology of the cell types is preserved throughout the procedure, isolated live cells change their shapes and tend to become spherical. Consequently, the microscopical examination fails to distinguish l-cells from early stages of nematoblasts and neuroblasts.

Interstitial stem cells and early committed precursors occur as large single cells and in pairs in maceration preparations. Those cells are known to form nerves (David and Gierer, 1974). Proliferating nematocytes occur in clusters of 4, 8, 16 and 32 cells (Sproull and David, 1979). Polyps of Hydra dissociated by the here described method yield suspensions of single cells. The appearance of cells in clusters by which they could be easily recognized is disrupted. It would be possible to separate big l-cells from small l-cells and nematoblasts by flow cytometric analysis and sorting on the basis of different cell sizes (time of light).

The cell type specificity of the Phytolacca-lectin has been successfully tested in the two species (H.attenuata and H.vulgaris), so far. Whether this holds true for other species remains to be investigated.

Cells sorted according to our method showed a viability of approximately 99%. The lectin was found to bind to both live and dead cells which cannot be distinguished from each other on the basis of their surface binding properties. In order to exclude the non viable cells from the flow sorting, dissociated cells could be stained simultaneously with propidium jodid which labels dead cells (Dangl et al., 1982; Yaross, 1986) and with FITC-lectin and be separated from each other by two-colour analysis (Sasaki et al., 1987).

Whenever large quantities of cells are to be sorted, their viability is reduced due to the circumstance that the cells have to be maintained in suspension for a relatively long period of time. A 0.9% NaCl solution (154 mM) was used as sheath fluid which leads to osmotical stress, since the dissociation medium for the hydra cells has a molarity of 93 mM. Therefore we had to sort the cells into a large volume of dissociation buffer in order to reduce the osmotic shock. It was also necessary to sort cells into a liquid medium, because cells are smashed at the tube wall, when sorted directly into the Eppendorf tube.

It proved to be mandatory to maintain the cell suspension at a low temperature, because incubation at 4°C prevents cross membrane reorganization and the internalization of lectin-ligand complexes (Dealtry and Sellens, 1987). When cells are sorted at room temperature for a period of more than one hour, the 90° scatter vs fluorescence cytogram changes significantly. This phenomenon can be explained by the uptake of the bound lectin into the cells and the subsequent changes of fluorescence intensities on the cell surface. Since only viable cells are able to reorganize membrane components, these findings further demonstrate the functional integrity of the dissociated cells.

The inherent tendency of lectins to agglutinate cells has limited their use as reagents for flow cytometry (Sharon, 1984). They are invariably polyvalent and therefore readily agglutinate cells bearing the appropriate cell surface sugars. Suspending the cells in the dense Percoll medium is therefore an important prerequisite for maintaining a suspension of single cells to avoid their agglutination during sorting.

A major disadvantage of this procedure was, that we were not able to obtain normal reaggregates and regenerates from neither labeled nor unlabeled cells which were recovered from the Percoll gradient. An interaction between the membranes and Percoll may possibly impair cell functions. Percoll was, however, seldom found to attach to cell surfaces as revealed by electronmicroscopical examination and membranes of Percoll-exposed cells were reported to exhibit normal properties (Pertoff and Laurent, 1977). It could well be that the labelling with FITC-conjugated lectin could impair the capability of the cells to reaggregate and regenerate. Percoll gradients normally are centrifuged at relatively low angular velocities (400-500 g) and for approximately 20-30 min. In our procedure we had to centrifuge the gradients at 2'000 g for 2 hr, in order to obtain a clear pre-separation of the different cell types with only minor cross-contamination between the interbands.

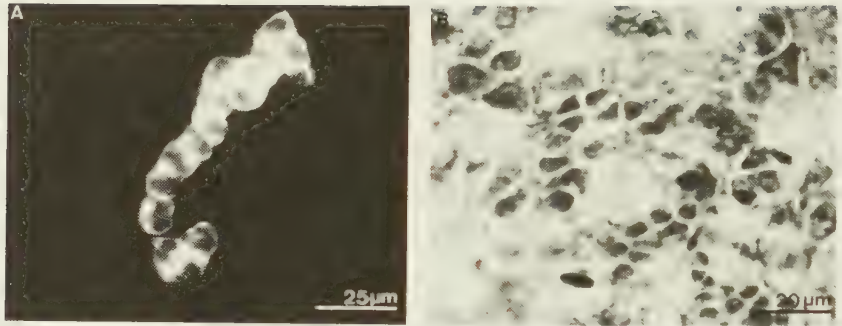


FIG. 1. Whole mounts of *Hydra*. A) Mount of an intact animal treated with FITC-lectin from *Phytolacca americana* showing a string like arranged cluster of nematoblasts. B) With toluidine blue stained small and big interstitial cells as well as nematoblasts.

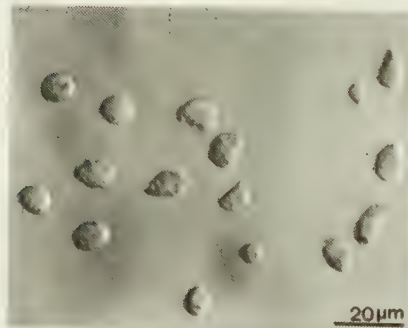


FIG. 2. Small and big interstitial cells as well as nematoblasts sorted from the interface of a 3-step Percoll density gradient with a density of 1.087-1.098 g/ml (Nomarsky illumination). Cells were sorted to 96% purity and 99% viability.

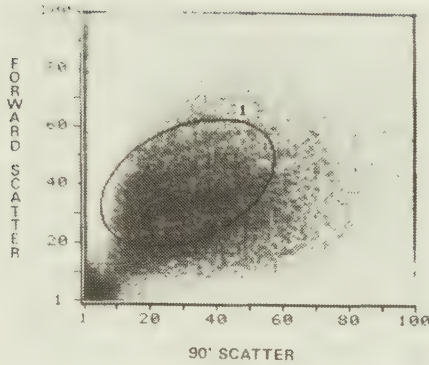


FIG. 3. Cytogram correlating forward scatter vs 90° scatter of the interface of the Percoll density gradient containing interstitial cells and nematoblasts to be sorted. The scatter signals were both measured in linear mode. In region 1 an analysis window was defined around the zone containing dense populations of cells.

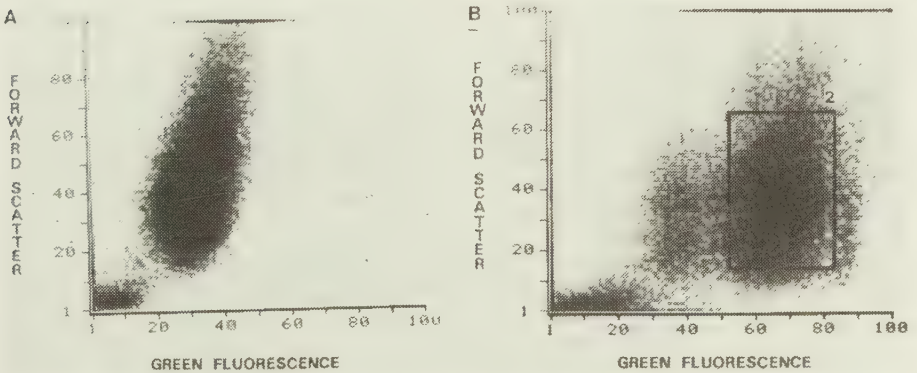


FIG. 4. Cytograms correlating forward scatter vs logarithmic green fluorescence. A) Populations of unlabeled cells (control). B) With *Phytolacca* lectin labeled populations of cells. A sorting window around the FITC-positive population was defined in region 2.

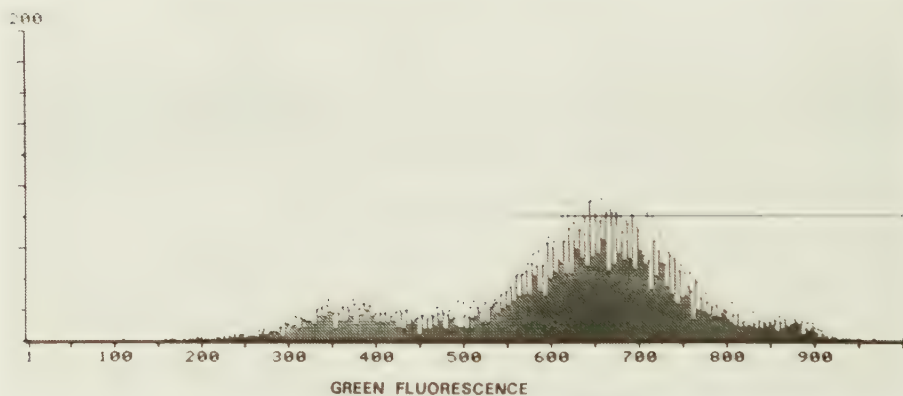


FIG. 5. Histogram of the fluorescence intensities of *Phytolacca* lectin labeled cell populations gated on region 1 (FIG. 3). Fluorescence was measured logarithmically. The negative control (unlabeled cells) exhibited only minor autofluorescence and was set to 0.4% positive cells. Approximately 70-80% of the total cell population were positively labeled. The ordinate represents relative cell numbers.

Table 1

Percentage of cell types present in the first interface^a of a 3-step Percoll density gradient

cell type	% of total cell count (mean + SD)
ecto- and endodermal epithelial cells	63.1 ± 2.4
interstitial cells and nematoblasts	26.3 ± 1.6
nerve cells	1.2 ± 0.01
stenotele nematocytes	1.2 ± 0.01
desmoneme nematocytes	7.0 ± 0.81
isorhizas	1.2 ± 0.11

^a interface between the 30 % and the 60 % (v/v) cushion with a density range from 1.049-1.075 g/ml.

Table 2

Percentage of cell types present in the second interface^a of a 3-step Percoll density gradient

cell type	% of total cell count (mean + SD)
interstitial cells and nematoblasts	75.9 ± 3.5
endodermal gland cells	6.6 ± 0.12
nerve cells	6.6 ± 0.76
stenotele nematocytes	1.3 ± 0.09
desmoneme nematocytes	8.5 ± 0.67
isorhizas	1.1 ± 0.2

^a interface between the 60 % and the 80 % (v/v) cushion with a density range from 1.087-1.098 g/ml.

Table 3

Percentage of cell types recovered from the bottom of the tube of a 3-step Percoll density gradient^a

cell types	% of total cell count (mean + SD)
stenotele nematocytes	48.4 ± 0.93
desmoneme nematocytes	35.4 ± 0.85
isorhizas	16.2 ± 0.36

^a the density at the bottom of the 80 % cushion was > 1.121 g/ml.

ISOLATION OF DIFFERENT CELL TYPES OF HYDRA ATTENUATA PALL.
(HYDROZOA, CNIDARIA) BY FLOW SORTING

ABSTRACT

Different cell types of Hydra were isolated by fluorescence activated cell sorting (FACS). The specific binding properties of the FITC-conjugated lectin from Bandeiraea simplicifolia were used for isolating ectodermal epithelio- muscular cells. The lectin from Dolichos biflorus served for the separation of endodermal gland cells and desmoneme nematocytes. The lectins from Psophocarpus tetragonolobus and Phytolacca americana allowed the isolation of desmoneme and stenotele nematocytes respectively. Nerve cells were isolated using the snake toxin α -Bungarotoxin and the antibody GT8. All cell types were sorted up to 90% purity and viability with the exception of desmoneme nematocytes which were always non viable as demonstrated by propidium jodid staining.

INTRODUCTION

The cellular architecture of the freshwater polyp Hydra attenuata (Hydrozoa, Cnidaria) is relatively simple. The tube like body column consists of an outer ectodermal and an inner endodermal cell layer both containing a limited number of cell types originating from three different stem cell lineages (Campbell and Bode, 1983).

The simplicity of these animals offers excellent opportunities for the investigation of morphogenetic processes at cellular and subcellular levels. A biochemical and molecular analysis of the control and pathway of differentiation of a specific cell type requires the availability of relatively pure populations of isolated cells. In the preceeding paper (see pages 9-21) we described a method for isolating viable interstitial stem cells (I-cells) and early stages of nematoblasts by fluorescence activated cell sorting (FACS). This method now at hand we took further attempts to isolate various cell types belonging to the other stem cell lineages. One of these lineages is represented by the epithelio-muscular cells of the ectoderm, from which the battery cells of the tentacles as well as the mucous-producing cells of the basal disk (Shostak and Globus, 1966; Campbell, 1967) derive. The epithelial cells of the endoderm which constitute another independent cell lineage function as digestive and muscular cells throughout the body column of the polyp. The third lineage is represented by the ectodermal interstitial cells, a rapidly proliferating multipotent population of stem cells which continuously give rise to the precursors of four types of nematocytes (Lehn, 1952; Slautterback and Fawcett, 1959), the nerve and sensory cells (Davis, 1974), the endodermal gland cells (Bode et al.; 1987) as well as gametes (Brien, 1961; Tardent, 1966).

Four different FITC-conjugated lectins as well as the snake toxin FITC- α -Bungarotoxin and one FITC-conjugated antibody were found to selectively label the particular cell types. These markers were used for the isolation of ectodermal epithelio-muscular cells, endodermal gland cells, nerve- and sensory cells and stenotele as well as desmoneme nematocytes.

MATERIAL AND METHODS

Culture Conditions

Polyps of Hydra attenuata Pall. were mass cultured at 14°C in a medium developed by Loomis and Lenhoff, 1956. The cultures were fed daily with freshly hatched larvae of Artemia. For all experiments animals were collected from the cultures not before 24 hr after the last feeding.

Dissociation Procedure

In all experiments polyps were dissociated into single cells according to the procedure described in detail on page 12. The dissociation medium contained 5 mM CaCl_2 , 2 mM MgSO_4 , 54 mM KCl, 20 mM Tris, 6 mM Na-Pyruvate, 6 mM Na_2 -Citrate, adjusted to pH 7.5 and Collagenase from Clostridium histolyticum (Serva Heidelberg, 0,74 U/mg, 5 mg/ml dissociation buffer) in a ratio of 8:1. Propidium jodid (Fluka, Buchs, Switzerland) staining was used to determine the viability of the cells following dissociation.

Labelling with FITC-Conjugates

Fluorescein-conjugated lectins from Bandeiraea simplicifolia (BS-1), Dolichos biflorus (Horse Gram), Phytolacca americana (Pokeweed), Psophocarpus tetragonolobus (Winged Bean) as well as α -Bungarotoxin from Bungarus multicinctus for labelling particular cell types were obtained from Sigma (St. Louis, USA). The antibody GT8 (IgG_3) was kindly supplied by

Prof. H. Bode (University of California, Irvine, USA). In each experiment approximately 1.8 ml of the cell suspension, each containing about 1.6×10^7 cells were added to 1.2 ml of one of the mentioned lectins (300 $\mu\text{g/ml}$ dissociation buffer), α -Bungarotoxin (100 $\mu\text{g/ml}$) or 200 μl GT8 respectively in a 15 ml Corning tube and incubated under constant gentle rotation for 10 min at room temperature. The labeled cell suspension was then immediately transferred to a Percoll density gradient. As a control for autofluorescence a batch of suspended cells was always incubated in 3 ml of dissociation buffer lacking FITC-conjugates. The specificity of the lectin- binding was assayed by the ability of the competing sugars to inhibit binding at concentrations of 50-100 mg/ml.

Coupling of FITC to the Antibody

FITC (Sigma) was coupled directly to the antibody. 100 μl of 25 mM FITC were added to 500 μl of antibody in a total volume of 1.5 ml PBS (pH 9). The coupling was performed overnight at 4°C under constant rotation. Unbound FITC was removed from the solution by washing 10 times with 2.0 ml PBS, each (pH 7) in a microconcentrator (Centricon-10, Amicon, Danvers, USA) by centrifugation (6000 rpm in a Sorvall RC-5B centrifuge, SS 34 rotor).

Percoll Density Gradient Centrifugation

Prior to the flow cytometric sorting the heterogeneous suspension of labeled cells were pre-separated on a three-step Percoll density gradient (Pharmacia, Uppsala, Sweden, 30%, 60%, 80% (v/v), 3 ml per step) according to the procedure outlined on page 13.

Enrichment of Endodermal Gland Cells

1 mg of Lectin from Phytolacca americana in 40 ml of distilled water was coupled to PVC plates (8 x 13 cm, Flow laboratories, Baar, Switzerland) under constant gentle motion for 2 hr. Unbound lectin was then removed by washing the plates with 200 ml distilled water and 200 ml dissociation buffer. Cells recovered from the second interband of a Percoll density gradient were diluted with dissociation buffer to a volume of 40 ml and incubated on the plate for 20 min under gentle motion. I-cells and nematoblasts stuck to the lectin covered bottom of the PVC plate and the cells in suspension which did not bind to the lectin were collected and concentrated by centrifugation at 500 x g for 10 min (Sorvall RT 6'000, H-1000B, swing-out rotor) and were resuspended in 2 ml of a 75% Percoll solution. All steps were performed on ice or at 4°C.

Whole Mounts

Polyyps of whole Hydra stained with FITC-conjugated lectins and antibody were mounted as outlined on pages 14-15. The second FITC-conjugated antibody was purchased from Antibodies Inc. (Davis, USA).

Microscopy

FITC- and propidium iodid-labeled cells as well as the whole mounts were examined with a Zeiss Fluorescence Microscope (Photomikroskop II, Zeiss, Oberkochen, FRG) equipped with phase contrast optics, a Reichert Jung Fluorescence Microscope (Polyvar, Vienna, Austria) and a Zeiss Axiophot, both provided with Nomarski illumination.

Flow Cytometry

A Cytofluorograf 50H (Ortho Diagnostic Systems, Inc., Westwood, MA) interfaced to a 2151 computer (Ortho Diagnostic Systems) and equipped with an argon-ion laser (Spectra Physics 164-05) operating at 488 nm and 100 mW was used for the analysis and the sorting of individual cell types. Forward small-angle light scatter and 90° scatter together with green fluorescence of 20'000 cells were analyzed. Sorting was maximized for optimal recovery and 3 droplets per cell were deflected. Cells were sorted at a flow rate of 1000 cells/s (see page 14).

RESULTS

Specific Labelling of Cell Types

The specific binding of different FITC-conjugated lectins to particular cell types of Hydra attenuata was investigated and demonstrated in whole mounts of the polyps which had been exposed to the lectins. The lectin from Bandeiraea simplicifolia was found to bind specifically to the surface of ectodermal epithelio- muscular cells (EMC's, Fig. 1a). The Dolichos biflorus-lectin showed less specific properties, since it was found to label both EMC's and endodermal gland cells. Figure 1b shows a typically labeled gland cell containing large vacuoles. As already demonstrated (see pages 15-16) the lectin from Phytolacca americana binds to interstitial cells (I-cells), nematoblasts and mature nematocytes, while the Psophocarpus tetragonolobus- lectin preferentially labels mature nematocytes (stenoteles,

desmonemes and isorhizas, Fig. 1c). The snake toxin α -Bungarotoxin (also FITC-conjugated) recognizes receptor sites of at least part of the nerve cells (Dvorak et al., 1978). However, the fluorescence of nerve cells labeled with this particular marker is limited to small areas on the cell surface. Consequently, in whole mounts of polyps no clearly visible labelling of nerve cells could be obtained with this label. On the other hand nerve cells of the hypostome and tentacles as well as early stages of nematoblasts could be selectively labeled with the FITC-antibody GT8 (kindly supplied by Prof.H.Bode, Irvine). However, the cell type specificity of the latter was found to be restricted to the particular clone of Hydra attenuata that we used in all our experiments. In other clones of the same species the GT8 antibody also binds to EMC's. Figure 1d shows a portion of a mounted tentacle featuring fluorescent GT8-labeled nerve cells.

In suspensions of single cells obtained by dissociation of polyps all of the above mentioned FITC-conjugated markers were found to bind indiscriminately to non-viable nematocytes (stenoteles, desmonemes and isorhizas) identified as such by propidium jodid staining (Dangl et al., 1982).

As a control for possible non-specific adsorption of the lectins, aliquots of the cell suspensions were incubated with both, the lectin and the competing sugar to which the lectin binds specifically. In such samples no labeled cells were found indicating that the lectin was completely bound to the sugar present in the medium.

The above described cell type specificities of the FITC-conjugated lectins, of the α -Bungarotoxin and the GT8 antibody constituted the methodical basis for the isolation of particular cell types in Hydra by FACS. The overall procedure required one or two preliminary steps by which an enrichment of the wanted cell type was achieved prior to flow sorting. One of these steps

consisted in the routinely performed Percoll density gradient centrifugation of the heterogeneous cell suspension. This preparation lead to an advanced but still incomplete separation of the various cell types which were collected from two interfaces of the gradient and at the bottom of the centrifuge tube. Unwanted cellular fragments and debris which had accumulated at the top of the centrifuge tube were discarded (see pages 16-17). The epithelio-muscular cells of both the ectoderm and endoderm were concentrated within the first interface at a density of 1.049-1.075 g/ml. The endodermal gland cells, the nerve cells and a few nematocytes, mostly desmonemes, could be recovered from the second interface (density range 1.087-1.098 g/ml), while the extremely dense (Weber et al., 1987) fully differentiated nematocytes (stenoteles, desmonemes and isorhizas) formed the pellet at the bottom of the tube (> 1.121 g/ml).

Isolation of Ectodermal Epithelio-muscular Cells

The EMC's which were specifically labeled with the FITC-conjugated lectin from Bandeiraea simplicifolia constituted part of the cells contained in the first interface collected after Percoll centrifugation. The cell suspension of this fraction was subjected to the flow cytometric analysis correlating forward angle light scatter vs 90° scatter. Figure 2a shows a three dimensional (isometric) projection of the typical negative control (unlabeled cell population) analyzed in an ungated cytogram correlating forward angle light scatter vs logarithmic fluorescence. Approximately 30-40% of the total cell populations analyzed were labeled. In Figure 3a the analysis of the lectin-labeled cell suspension is shown and the sorting window was defined around the region containing the pure EMC population which was sorted to 97% purity and 92% viability (Fig. 4a).

Isolation of Endodermal Gland Cells

After Percoll density centrifugation the endodermal gland cells were accumulated in the second interface together with I-cells, nematoblasts, nerve cells and some desmoneme nematocytes. Since the percentage of gland cells present in this fraction was small and therefore not suited for a flow cytometric isolation in reasonably large quantities, this cell type had to be enriched further in a second purification step. The latter took advantage of the fact that I-cells, nematoblasts and nematocytes specifically bind to the lectin from Phytolacca americana (see page 15). This lectin was coupled to the surface of PVC plates onto which the suspension of cells recovered from the second interface was poured. After incubation under gentle motion most of the I-cells and nematoblasts had bound to the lectin covered plate surface

and were discarded. In the recovered suspension of unbound cells the endodermal gland cells had undergone a 2-3 fold enrichment as compared to the relative frequency of this cell type in the second fraction following Percoll centrifugation.

After these two preliminary steps the cells were subjected to the flow cytometric analysis. Usually 2-5% of the total cell populations were FITC-positive. Figure 2b shows the isometric projection of the negative control and figure 3b of the Dolichos-labeled cell suspension featuring two regions containing different populations of labeled cells. The sorting windows were defined around region 1 which contained endodermal gland cells and region 2 which contained desmoneme nematocytes. Gland cells were sorted to 90% purity and 95% viability (Fig. 4b). The desmoneme nematocytes could be sorted to 97% purity but all cells of this type were exclusively non viable as demonstrated by propidium jodid staining.

Isolation of Nematocytes

The pellet at the bottom of the centrifuge tube that was collected following density centrifugation was found to contain exclusively nematocytes (stenoteles, desmonemes and isorhizas). Figure 2c shows the typical negative control (unlabeled population of cells) for this cell fraction.

1. Desmonemes: This cell type could also be sorted using the lectin from Psophocarpus tetragonolobus. The total populations of cells analyzed were positively labeled up to 97% and showed several regions of different populations of cells. The sorting window around region 1 in figure 3c contained 4% of the cells analyzed and the desmonemes sorted from this region were 95% pure but also not viable (Fig. 4c).

2. Stenoteles: This type of nematocyte could be sorted with the aid of both, the lectin from Psophocarpus tetragonolobus and the lectin from Phytolacca americana. Figure 3d shows the isometric projection of the cell suspension labeled with Phytolacca-lectin. The sorting window was defined around the region containing 1% of the cells analyzed. This region was found to contain stenoteles that could be sorted to 90% purity and viability (Fig. 4d). Cell suspensions labeled with Psophocarpus-lectin (see Fig. 3c) exhibited similar scatter and fluorescence characteristics as the Phytolacca-lectin labeled cells. The region containing the stenotele nematocytes was found in the same area of the Psophocarpus-cytogram as in that of the Phytolacca-cytogram. A sorting window of the same size was set around this region which again was found to contain 90% pure and viable stenoteles. The other populations of cells which exhibited fluorescence distinctly above control revealed to be mixtures of all three types of nematocytes. No region could be found that contained isorhizas only.

Isolation of Nerve Cells

Nerve cells were recovered from the second interface of the Percoll gradient. This type of cell could be sorted by means of two specific markers: a) the FITC-conjugated α -Bungarotoxin and b) the FITC-conjugated antibody CT8. In both cases 2-3% of the total cell populations were positively labeled. Figure 3e and f show the sorting windows around regions which contain the labeled nerve cells. Both included about 0.5-1% of the cells analyzed. As already mentioned, in addition to nerve cells, desmoneme nematocytes were also labeled and it was therefore not possible to find a region in the cytogram which did not contain this type

of cell. Nerve cells labeled with α -Bungarotoxin could be sorted to 90% purity and 99% viability (Fig. 4e). The nerve cells that were sorted with the antibody GT8 were to 80% pure. The contaminating cells were exclusively desmoneme nematocytes and early stages of nematoblasts. Since single live nerve cells which retract their processes and assume spherical shapes cannot be distinguished from equally spherical nematoblasts by morphological criteria it was impossible to determine the relative frequencies of both cell types present in the sorted material.

The FITC-conjugate was directly coupled to the antibody GT8. This showed to be necessary because the second antibody conjugated with high fluorescent FITC (goat-anti-mouse) that was used as the fluorescent label for GT8 was found to bind to all types of cells in the suspension. The negative control which consisted of cells incubated with the second antibody only exhibited the same fluorescence characteristics as cell populations which were labeled with first and second antibody.

DISCUSSION

The main categories of cells present in Hydra could be isolated in a live state and to a satisfactory degree of purity. The cell types sorted belong to two of the three stem cell lineages present in Hydra (ectodermal epithelio-muscular cells and interstitial cells). The endodermal epithelial cell which function as digestive cells constitute the third cell lineage. We have tested several antibodies (a gift from Prof.H.Bode, Irvine) in whole mounts and were able to find an antibody which is specific for these endodermal digestive cells. But as already described in the results for the antibody GT8, the second FITC-conjugated antibody was found to bind to all types of cells present in the suspension as revealed by flow cytometric analysis. We

also tried to couple FITC directly to the antibody but could not come up with satisfactory results in the labelling experiments.

The fluorescent markers used in the described experiments were found to bind to several types of cells in the heterogeneous suspension of single cells that was recovered from the Percoll density gradient. The analyzing cytograms feature several distinct regions containing populations of cells which exhibit different scatter and fluorescence characteristics indicating the presence of separate populations of different cell types. The microscopical investigation of cells sorted from these regions, however revealed mixtures of various cell types.

Therefore the regions from which the cell types in question could be sorted relatively pure and viable were small and contained only a limited percentage of the total of labeled cells in the suspension. We defined the sorting windows only precisely around the center of these populations in the cytogram in order to minimize contaminations by cells of other types.

All of the described FITC-conjugated markers were found to additionally bind to non viable nematocytes. Especially the markers we used to label nerve- and sensory cells tend to bind to desmonemes. Both of these cell types are of similar size and exhibit almost the same scatter and fluorescence characteristics. In order to sort nerve cells to a higher degree of purity, cells co-labeled with propidium jodid which stains the nucleus of dead cells and the FITC-marker could be distinguished from each other by two color analysis (Yaross, 1986; Sasaki et al., 1987). The propidium jodid labeled dead cells could be excluded from the sort and only viable FITC-positive cells would be obtained.

The method we developed for the dissociation of polyps includes a temperature and collagenase treatment. However, this treatment seems to exert a negative influence upon certain properties of the cell surface, e.g.

terminating the normal capability of the cells to reaggregate and form morphogenetically active regenerates. Further studies will have to determine, whether the receptors on the cell surface which are required for cell-cell-recognition and -interaction which seem to be damaged by either heat-or collagenase treatment, are able to regenerate in an appropriate 'in vitro' medium.

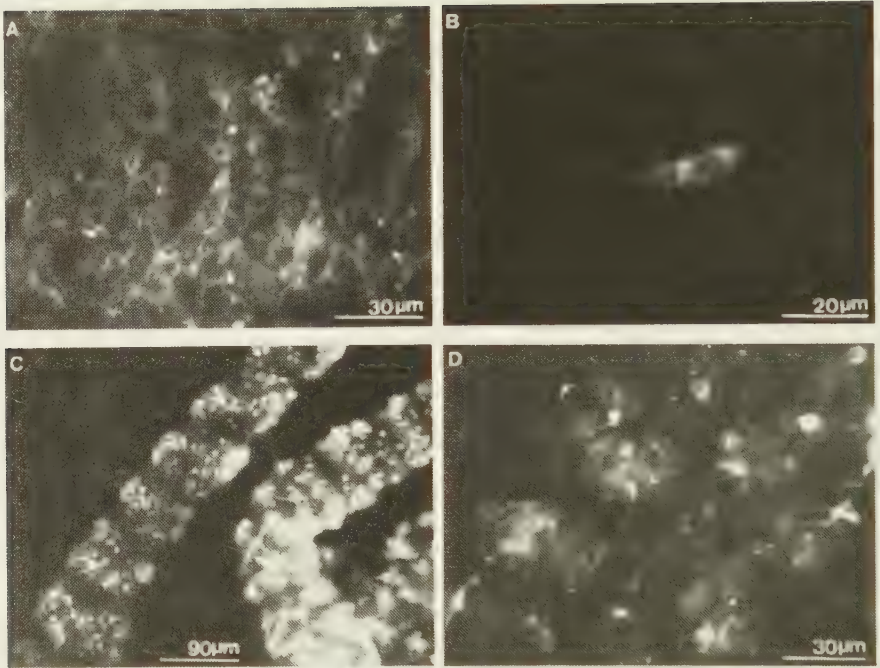


Fig. 1. Whole mounts of FITC-labeled *Hydra*. A) Portion of the body column of an intact animal treated with FITC-lectin from *Bandeiraea simplicifolia* showing labeled ectodermal epithelio-muscular cells (EMC's). B) With *Dolichos biflorus* labeled mount showing a typical endodermal gland cell containing large vacuoles. C) *Psophocarpus tetragonolobus* treated mounts with fluorescent nematocytes (stenoteles, desmonemes and isorhizas) of the tentacle. D) Antibody GT8 treated whole mount showing a portion of a tentacle featuring labeled nerve cells.

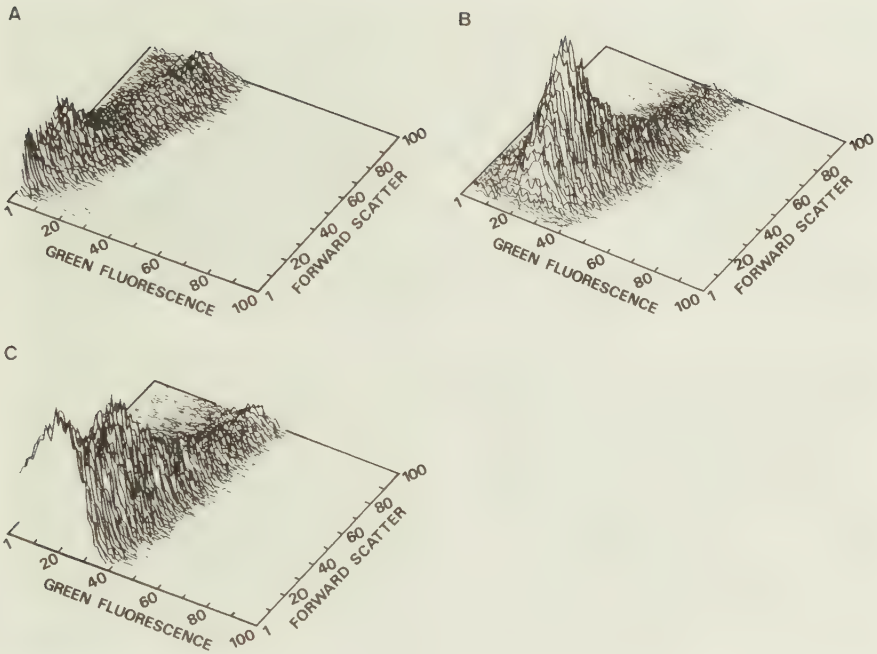
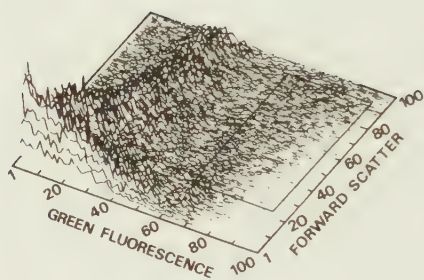
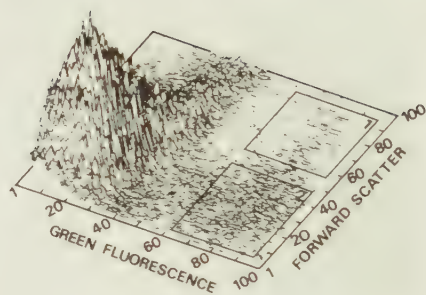


Fig. 2. Isometric projections of the negative controls (unlabeled cell populations) analyzed in ungated cytograms correlating forward angle light scatter vs logarithmic fluorescence. Cells were collected from two interfaces of a 3-step Percoll density gradient and at the bottom of the centrifuge tube. A) Fraction of cells collected from the first interface with a density range of 1.049-1.075 g/ml. B) Cells recovered from the second interface (density range 1.087-1.098 g/ml). C) Fraction of cells collected from the bottom of the centrifuge tube (density > 1.121 g/ml).

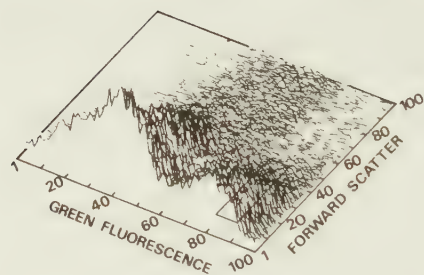
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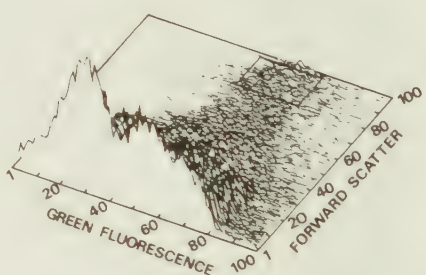
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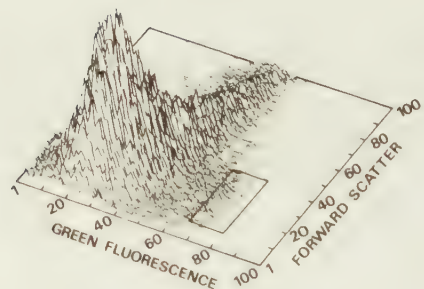
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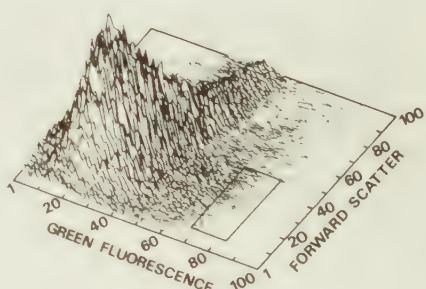
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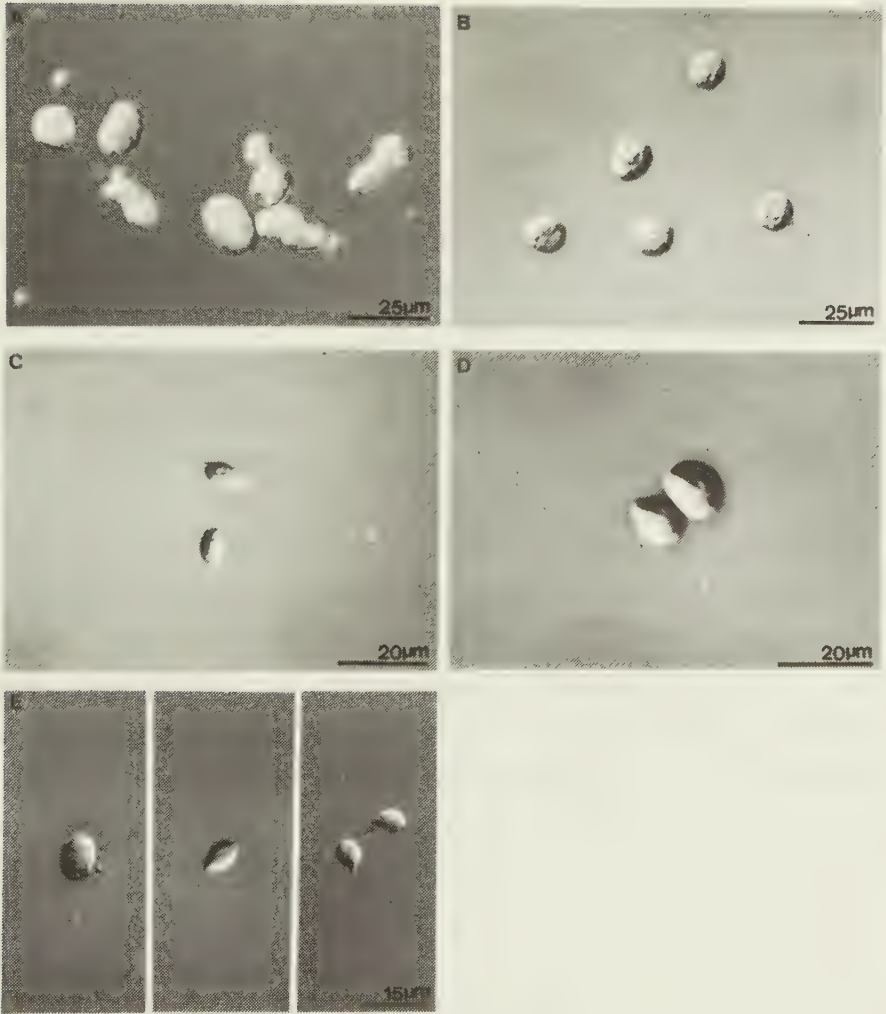


Fig. 4. Individual live cells as they appear after sorting. A) epitheliomuscular cells (EMC's). B) endodermal gland cells. C) desmoneme nematocytes. D) stenotele nematocytes. E) nerve cells.

LOCALIZATION OF THE HEAD ACTIVATOR OF HYDRA IN TISSUES, PARTICULAR CELL TYPES AND SUBCELLULAR FRACTIONS

ABSTRACT

Isolated ecto- and endodermal tissues, particular cell types as well as membrane- and cytosol preparations of Hydra (Hydrozoa, Cnidaria) were analyzed with respect to their content of head activator activity, a growth factor present in Hydra. Both, the ectoderm and the endoderm, contain head activator, with an activity recorded in the endoderm of 1/4 of that found in the ectoderm. The activity is restricted to extracts of nerve cells and epithelial cells of the ectoderm to 50%, respectively. No activity was recorded in the other cell types tested so far (interstitial cells and endodermal gland cells). 60% of the total activity found in extracts of whole animals was detected in a subcellular fraction which consisted of plasma membranes and endoplasmic reticulum. The remainder (40%) of the activity was found to be associated with the cytosol fraction.

INTRODUCTION

The maintenance of polarity, pattern formation in regenerating polyps of Hydra as well as cellular proliferation and differentiation are controlled by morphogenetic substances. So far, two sets of morphogens have been

isolated from polyps of the genus Hydra: an activator and inhibitor of head and bud formation and an activator and inhibitor of foot formation (Schaller et al., 1979; Schmidt et al., 1980).

The substance responsible for the initiation and promotion of head-specific growth and differentiation, the 'head activator', acts as a growth factor by stimulating cells which are arrested in the G₂-phase of the cell cycle to resume their mitotic activity (Schaller, 1976a). In addition, it activates the determination of uncommitted interstitial stem cells to differentiate into nerve cells (Schaller, 1976b; Holstein et al., 1986).

In Hydra 80-90% of the head activator activity was reported to be present in nerve cells (Schaller and Gierer, 1973; Grimmelikhuijzen and Schaller, 1979). However, it has been shown on the other hand that epithelial polyps which lack nerve cells, I-cells, nematocytes and gland cells (Diehl and Burnett, 1964; Campbell, 1976) produce normal buds and are able to regenerate normally respecting their original polarity, provided they are fed artificially. Surprisingly, such epithelial animals contain 8 times more head activator than normal polyps (Schaller et al., 1980a). The fact that all morphogenetic processes of the nerve free hydra occur in a normal fashion, raised some doubts as to the role of the nervous system in the control of differentiation and development. Schaller et al. (1980b) demonstrated that epithelial cells are capable of producing head activator (approximately 9%), but it seems that this property is repressed in the presence of nerve cells. All other cell types of the polyp are believed to contain no activity of this morphogen. So far, no information is available as to whether the ectodermal or endodermal epithelial cells or both are capable of producing the head activator.

According to Lentz (1965) and Schaller and Gierer (1973) 90-95% of the activity is structure-bound and only less than 10% are believed to be freely

diffusable. They isolated membranous particles which featured density characteristics that were similar to those of the endoplasmic reticulum. 70% of the activity was found to be present in this fraction.

In the present study we tried to gather more detailed information about the localization of the head activator by checking separately the ectoderm and the endoderm as well as by assaying particular cell types and subcellular fractions. We used the method described by Holstein et al. (1986) which is based on the fact that head activator stimulates the differentiation of I-cells to nerve cells as a system for assaying the activator activity. Consequently animals treated with extracts containing head activator show higher numbers of nerve cells.

The ecto- and endoderm were separated from each other in a live condition according to the method described by Epp et al. (1979). In addition, various cell types of both tissue layers were isolated by means of fluorescence activated cell sorting (FACS). Finally, we prepared subcellular fractions of membranes and cytosol and assayed these isolates for the presence of head activator.

MATERIAL AND METHODS

Culture Conditions

Polyps of Hydra attenuata were mass cultured in a synthetic medium (Loomis and Lenhoff, 1956) at 14°C and were fed daily with freshly hatched larvae of Artemia. For the isolation of both particular cell types and the subcellular components animals were collected randomly. Budless Hydra which

were used for the nerve cell assays were selected from the cultures not before 24 hr after the last feeding.

Separation of Ectoderm and Endoderm

To investigate whether the head activator is present either in one or in both tissue layers, the two were separated from each other according to the procedure described by Epp et al. (1979). Head and foot of the polyp were removed and the tubular body column was cut open longitudinally with microscissors. The specimens were incubated for 10 min in a medium containing a 1% solution of procain hydrochloride (Gattiker, Zurich, Switzerland) and culture medium in a ratio 2:1. Using a tungsten needle the endoderm could easily be pulled apart from the ectoderm.

Dissociation Procedure

For the isolation of individual cell types whole polyps were dissociated into single live cells according to the procedure described in detail on page 12. Approximately 200 polyps were incubated for 30 min at 37°C in 900 µl of a medium containing dissociation buffer (5 mM CaCl_2 , 2 mM MgSO_4 , 54 mM KCl, 20 mM Tris, 6 mM Na-Pyruvate, 6 mM Na_2 -Citrate, adjusted to pH 7.5) and Collagenase from Clostridium histolyticum (Serva Heidelberg; 0.74 U/mg, 5 mg/ml dissociation buffer) in a ratio of 8:1. Dissociation was achieved by gentle mechanical disruption with two complete passes through a Pipetman P-1000 pipettor fitted with a blue tip.

Isolation of Cell Types

Particular cell types (interstitial stem cells, endodermal gland cells and ectodermal epithelial cells) were isolated by flow cytometry according to the procedures outlined on pages 9-43. FITC-conjugated plant lectins from Phytolacca americana (Pokeweed), Dolichos biflorus (Horse Gram) and from Bandeiraea simplicifolia (BS-1), obtained from Sigma (St. Louis, USA) were used as cell type specific fluorescent markers. Enriched populations of unlabeled nerve cells were obtained by negative sorting out of a heterogeneous cell suspension in which all other cell types were FITC-lectin (Phytolacca-lectin and Dolichos-lectin) labeled. The dissociated cell suspension was incubated with the particular lectin (100 μ g in a total volume of 3 ml dissociation buffer) under constant gentle rotation for 10 min at room temperature. Prior to cell sorting labeled cells were pre-separated on a three-step Percoll density gradient (Pharmacia, Uppsala, Sweden, 30%, 60%, 80% (v/v), 3 ml per step). Cells were analyzed and sorted with a Cytofluorograf 50H (Ortho Diagnostic Systems Inc., Westwood, MA) interfaced to a 2151 computer (Ortho Diagnostic systems) and equipped with an argon-ion laser (Spectraphysics 164-05) operating at 100 mW and 488 nm. Forward small-angle light scatter and 90° scatter together with green fluorescence of 20'000 cells were analyzed. Sorting was maximized for optimal recovery and 3 droplets per cell were deflected. Cells were sorted at a flow rate of 1000 cells/s.

Tissue Homogenization

For the isolation of cytosol and membranes approximately 10'000 polyps were collected from the mass cultures and washed three times in homogenization buffer prepared according to Maeda et al. (1983) with minor modifications (1 mM $MgCl_2$, 30 mM NaCl, 1 mM dithiothreitol, 0.003 mM benzamidine hydrochloride, 0.02% NaN_3 , 100 μg of DNAase/I, pH 7.4). The animals were minced on ice with a razor blade, transferred to a homogenizer (DuPont Instruments) fitted with a teflon pestle and filled up to a volume of 14 ml with homogenization buffer. Homogenization was achieved by five slow up-and-down movements at a pestle speed of 2800 rpm (power setting of 3).

Isolation of Cytosol

The homogenates of entire hydra were transferred to a 12 ml centrifuge tube (Beckman, USA) and centrifuged at 166'000 x g in a Sorvall ultracentrifuge (OTD65B, type 65 rotor) for 1 h. The clear cytosolic supernatant was collected from the top with a pasteur pipet.

Isolation of Cell Membranes

Cell membranes were isolated according to the procedure outlined by Maeda et al. (1983). 14 ml of the homogenate were layered on top of 10 ml of a 41% solution of sucrose dissolved in homogenization buffer. This preparation was centrifuged at 95'000 x g in a Sorvall ultracentrifuge (OTD65B, Beckman swinging-bucket rotor, type SW 27 rotor) for 1h. The white interfacial band of membranes was collected. Upon dilution of the sucrose with 30 ml of

homogenization buffer, the membranes were pelleted by centrifugation at $95'000 \times g$ for 20 min, washed twice and stored at -80°C .

Enzyme Assays

The activity of the marker enzymes specific for the various cell organelles were used to investigate the purity of the isolated subcellular fractions. The following markers were used: lactic dehydrogenase for the cytosol (Vassault, 1983); alkaline phosphodiesterase I for plasma membranes (Razzell, 1963); glucose-6-phosphatase for endoplasmic reticulum (Swanson, 1955); succinate-cytochrome-c reductase for mitochondria (Fleischer and Fleischer, 1967) acid phosphatase for lysosomes (Moss, 1984) and catalase for peroxisomes (Aebi, 1983).

Preparation of Crude Extract

About 1500 Hydra were sonicated in 3 ml of hydra medium for 3 min with a Branson sonifier (B-15P) at 60 W on ice cooling. The protein content of each extract was determined according to the method described by Schachterle et al. (1973). One hydra (without buds) has an average protein content of 23 $\mu\text{g}/\text{ml}$. All concentrations of the extracts tested were based on the protein content of 1 hydra.

Preparation of Methanol Extract

Methanol extracts were prepared as outlined by Holstein et al. (1986). Crude extracts of the various samples were prepared by sonication in 5 ml of hydra medium for 3 min. The protein content of the crude extract was

determined and the volume was adjusted to 10 ml with culture medium. After addition of 10 ml methanol, the extract was shaken vigorously and centrifuged at 5000 x g (6500 rpm on Sorvall RC-5B, SS 34 rotor) for 15 min. The supernatant was decanted and saved. The pellet was resuspended by sonication in 10 ml methanol, shaken again, and centrifuged at 5000 x g for 15 min. The extraction procedure was repeated three times. All supernatants were then pooled and centrifuged again at 5000 x g for 15 min to remove residual precipitated material. The final supernatant was extracted 4 times with 10 ml petroleum ether (bp 30-60°C). The methanol extract was concentrated completely on a rotary evaporator, adjusted to a final volume of 5 ml (i.e. the starting volume) with culture medium and was stored at -80°C.

Nerve Cell Assay

Committed nerve precursors were assayed according to Holstein et al. (1986). Animals were injured with three well-spaced transverse cuts along the body column and were incubated for 18 hr in a 6-well costar plate at a density of 10 animals per 10 ml of hydra medium. In all experiments 60 animals were macerated and analyzed for each concentration. Control animals were injured and incubated in culture medium only.

Maceration Procedure

For the identification of cell types and the determination of the relative numbers of nerve- and epithelial cells 10 animals were macerated in 200 µl of a solution containing acetic acid, glycerin and water in a ratio of 1:1:26 (David, 1973) for 1 day at 4°C. The dissociation into single cells was

achieved by gentle shaking and the cells were subsequently fixed with 20 μ l of a 4% formalin solution. 10 μ l of the macerate was placed on a microscope slide, spread over an area of about 1 cm² with a fishing line and dried in an exicator. Three microscope slides were analyzed per sample. Cell counts were done with an Olympus phase contrast microscope. Approximately 50-120 nerve cells and 600-660 epithelial cells (ectodermal and endodermal epithelial cells) were counted per sample.

RESULTS

Crude extracts as well as methanol extracts prepared from the various samples (whole polyps, separated ecto- and endoderm, isolated cell types and isolated membranes and cytosol) were tested for their head activator activity according to the method described by Holstein et al. (1986). This assay measures the degree of activity by determining quantitatively the activator dependent increase of the nerve cell population in the test polyps as compared to control animals. The number of nerve cells counted in maceration preparations (David, 1973) were normalized to the total number of both ectodermal and endodermal epithelial cells present (Nv/Epi). In the controls the average ratio of Nv/Epi usually ranged between 0.09 and 0.11. However, during the course of this work, a long-term shift of this value up to 0.125 was observed, which was probably due to uncontrollable changes of the culture conditions. All figures recorded above the mentioned control values were considered to be the consequence of the head activator activity present in the tested extracts. The maximal values of Nv/Epi ranged between 0.16 and 0.17. The activity was expressed as % activation over

control animals. The increasing concentrations of the extracts tested were based on the protein content of one Hydra without buds (approx. 23 µg/ml).

Extracts of Whole Polyps and of Isolated Ectoderm and Endoderm

The maximum increase in Nv/Epi value obtained after incubation with crude extracts prepared from whole polyps was equivalent to concentrations of 1-2 hydra/ml. Any further increase of the extract concentration resulted in a decrease of the overall activity down to less than the control levels (also see Holstein et al., 1986). Crude extracts of whole animals caused an increase of approximately 60% of the nerve cell population within 18 hr. While equivalent extracts of the ectoderm caused a 40% increase, extracts prepared from the endoderm were less active and lead to a 10% increase only. The results are shown in figure 1. They clearly indicate that the morphogen is present in both the ectoderm and the endoderm of the polyps body column. The activities found in both tissue layers did not add up completely to the total activity (60%) found in extracts of whole animals. This discrepancy may be attributed to the following circumstances: For the separation of the ecto- and endoderm the head (hypostom and tentacles) and the pedal disk had to be removed, thus, the extracts prepared from whole polyps contained components of these structures which were lacking in the tissue isolates. In addition, the separation of the two tissue layers required a treatment with 0.7% Procain hydrochloride (10 min) which, although diluted, was still present in the extract to be tested and may have exerted a slight inhibition upon either stem cell commitment or differentiation.

This second hypothesis was tested by incubating whole polyps in a 0.7% Procain solution for 10 min without removing their heads and basal disks. The extracts of these Procain treated whole animals exhibited an activity

which revealed to be about 10% reduced as compared to extracts of untreated whole polyps (Fig. 2), thus, confirming the interpretation expressed above. The purification of crude extracts by means of methanol extraction for isolating peptides and other low molecular weight molecules resulted in an approximately 3-fold increase of the activity as compared to that exhibited by crude extracts. In this case, the maximal Nv/Epi values were obtained at concentrations corresponding to 0.1-0.5 hydra/ml. Essentially all of the biological activity present in the crude extracts could be recovered in the methanol phase. The range within which methanol extracts stimulate nerve cell commitment is smaller than that of the crude extracts.

The comparison of methanol extracts with crude extracts prepared from isolated tissues indicates that the activities in methanol extracts are slightly higher (15% and 45% for endo- and ectoderm respectively), than in crude extracts and now added up to the value of 60% (Fig. 3). During the methanol extraction the Procain required for the separation of the two cell layers was removed and consequently no further inhibiting effect was observed.

Extracts of particular cell types

Thanks to our newly developed method for flow cytometric isolation of viable individual cell types we were able to isolate ectodermal epithelial cells, endodermal gland cells and interstitial stem cells together with early stages of nematoblasts to more than 90% purity and in quantities large enough to allow methanol extraction of particular cell types and the subsequent analysis of their head activator activity in the nerve cell commitment assay.

Nerve cells were enriched by negative sorting. In this isolation procedure all cell types of the heterogenous cell suspension other than nerve cells were

FITC-lectin labeled (endodermal gland cells, I-cells and nematocytes). Only the non-fluorescent cells were sorted and 85% were found to be nerve cells. Contaminating cells were I-cells and nematoblasts which had failed to react with the cell type specific lectin and therefore were not fluorescently labeled.

Methanol extracts of all samples of individual cell types were tested with respect to their head activator activity. The extract prepared from isolated nerve cells was found to cause an approximately 30% increase of the NV/Epi value. On the other hand the extract from ectodermal epithelial cells revealed to contain an equal activity as the nerve cells, since their Nv/Epi value was also found to show a 30% increase. Both percentages add up to the total of 60%, which was also obtained by incubation with extracts from whole animals (Fig. 4). The extracts prepared from the interstitial cells and nematoblasts which were isolated with the specific FITC-conjugated lectin to 96% purity caused no activation in the assay indicating that no head activator is present in these cell types. Therefore it can be assumed that the presence of these two types of cells in the negative sorted fraction of nerve cells (less than 15%) had no influence on the activator activity found in nerve cells. Extracts of endodermal gland cells were also found to contain no detectable activator activity.

So far, we failed to find a way to isolate endodermal epithelial cells (digestive cells). However, the results obtained by the analysis of the isolated endoderm suggest that in this cell layer no cell type other than nerve cells is responsible for the activity found. According to Smid (1984) the ectoderm of Hydra attenuata contains twice as many nerve cells as the endoderm. Therefore the head activator activity recorded in endodermal extracts, which is 1/4 of that found in ectodermal preparations, could well be attributed to the total population of endodermal nerve cells. Assuming

that the activity in the ectoderm is due to twice as many nerve cells only half of the total activity found in this tissue layer could be obtained, leaving the other half to another cell type. As the present study has shown, detectable head activator activity is limited to nerve cells of both the ecto- and the endoderm and to epithelio-muscular cells of the ectoderm.

Extracts of Subcellular Components

In order to localize the head activator activity at the cellular level we isolated pure fractions of cytosol and membranes as outlined in the chapter material and methods. To check for the presence of the different cell organelles in the isolated fractions we assayed the fractions with particular marker enzymes. The organelles, the enzymes and their specific activities are listed in table 1 for the cytosolic fraction. Table 2 stands for the membrane fraction. The latter turned out to be a mixture of plasma membranes and endoplasmic reticulum. The methanol extract prepared from this fraction caused a 30% increase in *Nv/Epi* ratio. This increase alone accounts for approximately 60% of the total activity that was recorded in extracts of whole polyps (Fig. 5). This finding is in good agreement with the figures given by Schaller and Gierer (1973) who reported that 70% of the head activator activity was associated with membranes. So far, all our attempts aiming at a separation of the plasma membranes from the endoplasmic reticulum have failed. For this reason we are unable to determine whether the head activator is associated with either of the two or with both structures.

The cytosol could be isolated without contaminations by other cell organelles and caused an increase in *Nv/Epi* ratio of 25% which accounts for approximately 40% of the total activity found in Hydra (Fig. 5).

DISCUSSION

Suspensions of live single cells obtained by dissociation of hydra can be reaggregated and will regenerate normal polyps. In the course of this regeneration process the head will always form where cells from the head region are located indicating that the factors which control morphogenesis in Hydra survive dissociation into suspensions of single cells (Gierer et al., 1972). These suspensions of dissociated cells were found to contain normal amounts of head activator (Schaller, 1975) which would mean that at least most of this morphogenetic substance is retained within the cells and not in the intercellular space. This fact was crucial for all experiments described in this paper which aimed at a stepwise localization of the head activator in isolates of the various structural components of the polyps.

As a test for the presence and the degree of activation caused by the morphogen we used the assay recently described by Holstein et al. (1986) which proved to be most reliable. Indeed, the results of our preliminary tests, in which crude- and methanol extracts of whole hydra were assayed, perfectly matched those of these authors. A second proof for the reliability of the test was given by the experiments in which the ectodermal and endodermal cell layers were assayed separately. They not only showed that the head activator is present in both layers but the figures obtained from each isolate added up perfectly to the activity recorded in extracts of whole polyps.

The experimental series in which extracts of particular cell types were tested also led to results, which clearly proved that ectodermal epithelio-muscular cells and the nerve cells of both the ecto- and endoderm

alone harbour the head activator. Since, so far, we did not find a specific marker for the isolation of endodermal epithelial cells (digestive cells) by flow cytometry, this particular cell type could not be assayed for head activator activity. However, the tests with isolated cell layers, which had clearly shown that extracts of the endoderm are considerably less active than those of the ectoderm, suggest that the head activator activity recorded in the endodermal isolates is restricted to the nerve cells present in this layer and not to the digestive nor to the gland cells.

Our findings concerning the cell type specific localization of the head activator activity are partly in agreement with those published by other authors (Schaller and Gierer, 1973; Grimmelikhuijzen and Schaller, 1979) who located 80-90% of the activity in nerve cells which they had enriched on glycerol gradients. Their fraction containing the highest activity was found to contain 50% nerve cells and at least 35% epithelial cells. This could be interpreted that at least part of the activity was due to the presence of the epithelial cells, even though another fraction which contained 50% of this cell type was less active. In another attempt Schaller et al. (1980b) enriched epithelial cells of normal hydra on a Percoll density gradient but could only recover 9% of the activity.

The fractions of cells that we were able to obtain by flow sorting contained viable epithelio-muscular cells of the ectoderm to more than 90% and nerve cells to more than 85% purity. The suspension of individual nerve cells were free of contaminations by epithelial cells. Our findings that nerve cells and epithelial cells contain approximately equal amounts of head activator activity could be due either to the better isolation procedure or to the possibility that the clone of Hydra attenuata we used for all experiments feature a different distribution pattern of the morphogen, even though our cell counts yielded a normal proportion between nerve- and epithelial cells.

Schaller and Gierer (1973) have demonstrated, that 70% of the head activator activity is present in membraneous structures having density characteristics similar to endoplasmic reticulum and that 90-95% of the activity is structure bound, i.e. sedimentable. In the present study we assayed isolated fractions of membranes and of cytosol. Using the marker enzymes specific for various cell organelles, we were able to determine the composition and purity of two fractions which were found to contain head activator activity. Their respective activities added up to the total activity recorded in extracts of whole polyps.

The 60% activity found in association with plasma membranes and endoplasmic reticulum are consistent with the results obtained by the above mentioned authors. In contrast, the 40% activity exhibited by our cytosol preparation is far higher than the proposed less than 10% of freely diffusable and not structure-bound head activator.

Unfortunately all attempts to separate plasma membranes and endoplasmic reticulum which were contained in the same fraction from each other have failed, so far. These two subcellular components have overlapping density characteristics which prevent their separation by means of gradient centrifugation. In addition, many components of the endoplasmic reticulum as determined by the specific enzyme activities are also located in plasma membranes, in lysosomes and in the golgi apparatus (Jarasch et al., 1979). The activity of acid phosphatase (a specific marker enzyme for the lysosomes) that we detected in our membrane preparation was probably due to the fact that this enzyme is easily released from injured granules, thus contaminating the membrane fraction. As far as the presence of the golgi apparatus in the membrane preparation is concerned we were not able to quantitate this component because no enzyme activity of the known marker enzymes could be recorded.

The results described in this paper indicate the limitation of the attempts to localize the head activator in association with cellular components by means of subcellular fractionation. Various antibodies against this morphogen have become available and further investigations on the localization of the morphogen should be done by immunohistochemical methods. However, as Bodenmüller and Roberge (1985) report, these attempts have failed so far, because the head activator can not be fixed by conventional fixation procedures. This was shown by the detection of the activator in the fixation medium after tissue fixation.

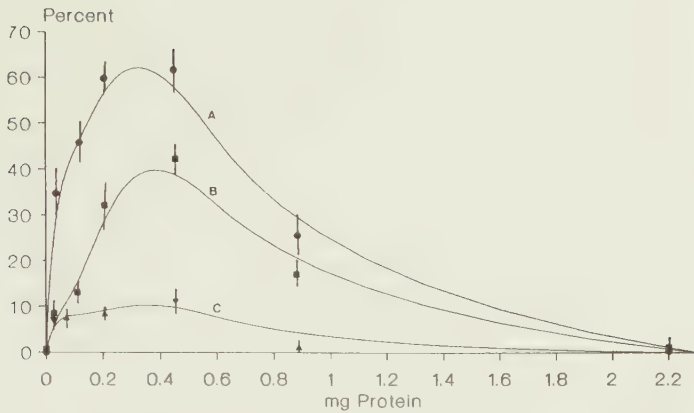


Fig. 1. Head activator activity in crude extracts of whole hydra (A), isolated ectoderm (B) and endoderm (C) on the differentiation of committed l-cells to nerve cells. Whole animals were injured with three well-spaced transverse cuts and treated with crude extracts for 18 hr. Afterwards they were macerated and the relative numbers of nerve cells normalized to the number of epithelial cells (Nv/Epi) were determined. Each point represents the analysis of 60 animals and approximately 300 nerve cells and 3700 epithelial cells were counted. The activity is given in % increase of Nv/Epi over control animals. Extract concentration is given in mg protein based on the protein content of 1 hydra/ml (23 μ g/ml).

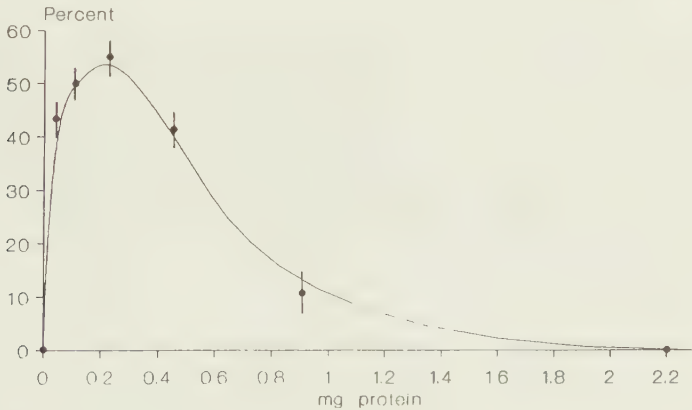


Fig. 2. Head activator activity in crude extract of whole hydra which were incubated in 0.7% Procain hydrochloride for 10 min prior to homogenization on the differentiation of committed l-cells to nerve cells. Whole animals were injured with three well-spaced transverse cuts and treated with crude extract for 18 hr. Afterwards they were macerated and the relative numbers of nerve cells normalized to the number of epithelial cells (Nv/Epi) were determined. Each point represents the analysis of 60 animals and approximately 300 nerve cells and 3700 epithelial cells were counted. The activity is given in % increase of Nv/Epi over control animals. Extract concentration is given in mg protein based on the protein content of 1 hydra/ml (23 μ g/ml).

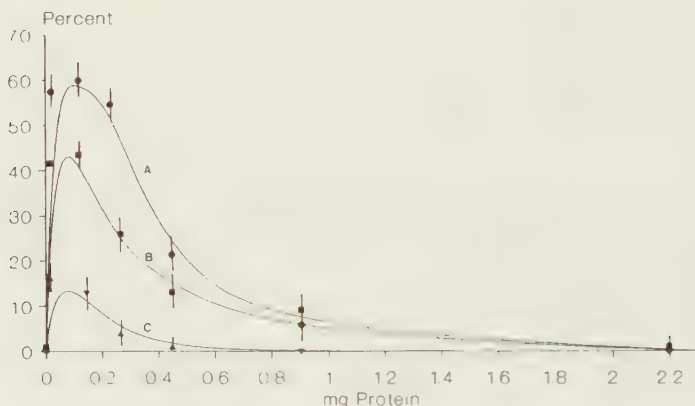


Fig. 3. Head activator activity in methanol extracts of whole hydra (A), isolated ectoderm (B) and endoderm (C) on the differentiation of committed l-cells to nerve cells. Whole animals were injured with three well-spaced transverse cuts and treated with the extracts for 18 hr. Afterwards they were macerated and the relative numbers of nerve cells normalized to the number of epithelial cells (Nv/Epi) were determined. Each point represents the analysis of 60 animals and approximately 300 nerve cells and 3700 epithelial cells were counted. The activity is given in % increase of Nv/Epi over control animals. Extract concentration is given in mg protein based on the protein content of 1 hydra/ml (23 μ g/ml).

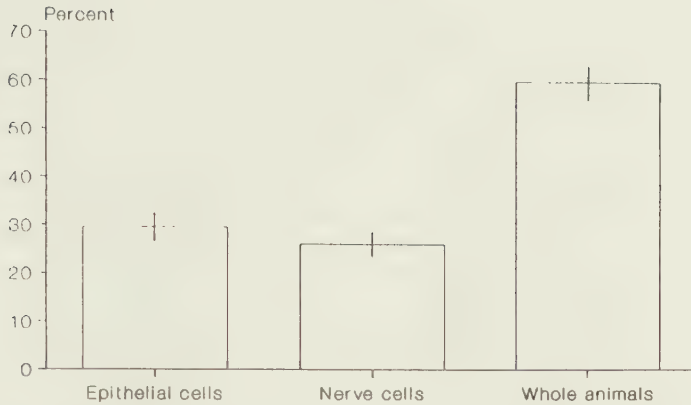


Fig. 4. Head activator activity in methanol extracts of isolated epithelial- and nerve cells compared with the activity present in extracts of whole hydra on the differentiation of committed l-cells to nerve cells. Whole animals were injured with three well-spaced transverse cuts and treated with the extracts for 18 hr. Afterwards they were macerated and the relative numbers of nerve cells normalized to the number of epithelial cells (Nv/Epi) were determined. The activity is given in % increase of Nv/Epi over control animals.

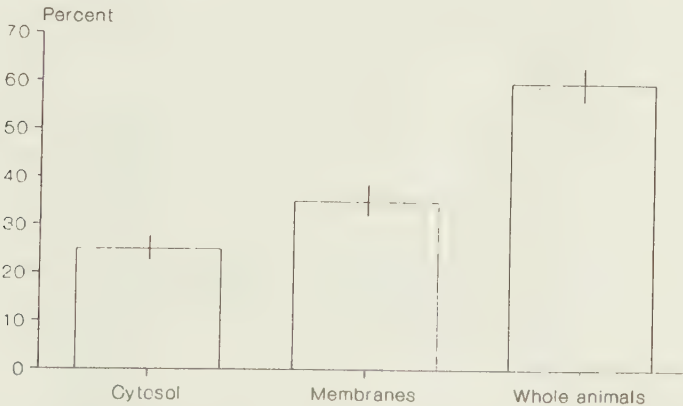


Fig. 5. Head activator activity in methanol extracts of isolated cytosol and membranes compared with the activity present in extracts of whole hydra on the differentiation of committed l-cells to nerve cells. Whole animals were injured with three well-spaced transverse cuts and treated with the extracts for 18 hr. Afterwards they were macerated and the relative numbers of nerve cells normalized to the number of epithelial cells (Nv/Epi) were determined. The activity is given in % increase of Nv/Epi over control animals.

Table 1
Marker enzymes analyzed in the cytosol preparation of Hydra

cell organelle	enzyme	enzyme activity in	
		homogenate	cytosol
plasma membranes	alkaline phosphodiesterase I	0.039	-
endoplasmic reticulum	glucose-6-phosphatase	0.19	-
mitochondria	succinate-cytochrome c reductase	0.262	-
lysosomes	acid phosphatase	1.31	0.20
peroxisomes	catalase	0.013	-
cytosol	lactate dehydrogenase	0.263	0.229

Hydra tissue was homogenized and the cytosol was prepared as described in Material and Methods. The purity of the isolated cytosol was determined with the marker enzymes of the various cell organelles. The enzymes were assayed in crude homogenates and in the cytosol preparation. Specific activities are given in $\mu\text{mol/h}$ per mg protein.

Table 2
Marker enzymes analyzed in the membrane preparation of Hydra

cell organelle	enzyme	enzyme activity in	
		homogenate	membranes
plasma membranes	alkaline phosphodiesterase I	0.031	0.045
endoplasmic reticulum	glucose-6-phosphatase	0.020	0.02
mitochondria	succinate-cytochrome c reductase	0.020	-
lysosomes	acid phosphatase	1.900	9.17
peroxisomes	catalase	0.015	-
cytosol	lactate dehydrogenase	0.230	-

Hydra tissue was homogenized as described in Material and Methods. Plasma membranes were prepared according to the method outlined by Maeda et al. (1983). The purity of the isolated membranes was determined with the marker enzymes of the various cell organelles. The enzymes were assayed in crude homogenates and in the membrane preparation. Specific activities are given in $\mu\text{mol/min}$ per mg protein.

ZUSAMMENFASSUNG

1. Ziel dieser Arbeit war die Lokalisation der Kopfaktivator-Aktivität von Hydra attenuata (Hydrozoa, Cnidaria) in den voneinander getrennten Zellschichten (Ektoderm, Entoderm), in verschiedenen isolierten Zelltypen, sowie in subzellulären Fraktionen (Membranen, Cytosol).

2. Für den qualitativen und quantitativen Nachweis der Aktivität des Aktivators wurde dessen Eigenschaft, die Differenzierung von I-Zellen zu Nervenzellen zu induzieren, herangezogen. Die Testtiere wurden zu diesem Zweck in den zu analysierenden Roh- oder Methanolextrakten inkubiert. In den anschliessend angefertigten Mazerationspräparaten wurde die Zahl der Nervenzellen ermittelt und mit jenen von unbehandelten Kontrolltieren verglichen.

3. Die Inkubation mit Rohextrakten von ganzen Polypen führte zu einer Erhöhung der Nervenzellzahl um 60%, während die Extrakte von isoliertem Ektoderm eine Erhöhung von 40%, jene von Entoderm eine solche von 10% zur Folge hatten. Dies bedeutet, dass der Kopfaktivator in beiden Zellschichten vorhanden ist.

4. Ekto-und Entoderm zusammen ergaben eine etwas geringere Aktivität als die Extrakte von ganzen Polypen. Diese Unstimmigkeit ist vermutlich darauf zurückzuführen, dass die für die Trennung der beiden Gewebeschichten erforderliche Inkubation in 0.7% Procain eine schwach negative Wirkung auf die Aktivität ausübt. Zur Kontrolle dieser Hypothese wurden ganze Polypen

in der Procainlösung inkubiert und anschliessend getestet. Das Ergebnis zeigte eine um ca 10% geringere Aktivität.

5. Methanolextrakte von ganzen Tieren führten zur gleichen Erhöhung der Nervenzellzahl wie die Rohextrakte ganzer Polypen. Im Fall der voneinander getrennten Zellschichten war die Aktivität von Methanolextrakten um ca 5% höher als jene der Rohextrakte. Bei der Extraktion mit Methanol konnte das Procain entfernt und damit dessen inhibitorische Wirkung ausgeschlossen werden.

6. Für die Isolation der verschiedenen Zelltypen von Hydra musste zunächst eine neue Dissoziationsmethode entwickelt werden, die eine nahezu vollständige Desintegration der Polypen in lebende Einzelzellen erlaubte und ausserdem eine typspezifische Markierung der Zellen ermöglichte. Die heterogenen Zellsuspensionen wurden in einem Percoll Stufengradienten vorgetrennt, wodurch eine bis zu dreifache Anreicherung der einzelnen Zelltypen erzielt werden konnte.

7. Die verschiedenen Zelltypen wurden mit FITC-konjugierten Lektinen, dem Schlangengift α -Bungarotoxin und einem direkt FITC-konjugierten Antikörper spezifisch markiert, wobei sich folgende Affinitäten als für das Trennungsverfahren praktikabel erwiesen.

Zelltyp	Marker
Interstitielle Zellen	Lektin von <u>Phytolacca americana</u>
Ektodermale Epithelzellen	Lektin von <u>Bandeiraea simplicifolia</u>
Entodermale Drüsenzellen	Lektin von <u>Dolichos biflorus</u>
Stenotelen	Lektin von <u>Phytolacca americana</u>
	Lektin von <u>Psophocarpus tetragonolobus</u>
Desmonemen	Lektin von <u>Dolichos biflorus</u>
	Lektin von <u>Psophocarpus tetragonolobus</u>
Nervenzellen	Toxin: α -Bungarotoxin
	Antikörper GT8

Nach Anreicherung wurden die FITC-markierten Zellen durchflusszytometrisch aus den heterogenen Zellsuspensionen isoliert, wobei ein Reinheitsgrad von 90% erreicht wurde. 90% der isolierten Zellen waren lebend.

8. Methanolextrakte der getrennten Zelltypen wurden im Bioassay auf ihre Aktivator-Aktivität hin untersucht. In den Extrakten von I-Zellen und entodermalen Drüsenzellen konnte keine Aktivität nachgewiesen werden. Solche von Nervenzellen und ektodermale Epithelzellen führten in den Testpolyphen je zu einer ca 30% Erhöhung der Nervenzellzahl. Die Aktivitäten beider Zelltypen zusammen ergaben die maximale, in Rohextrakten von ganzen Polypen gefundene Aktivität (60%). Entodermale Epithelzellen konnten bisher noch nicht isoliert werden.

9. Cytosol und Membranen wurden isoliert und mit den jeweiligen, für die verschiedenen Zellorganellen typischen Leitenzymen auf ihren Reinheitsgrad hin untersucht. Das Cytosol konnte rein isoliert werden, wohingegen die Fraktion von Membranen endoplasmatisches Retikulum sowie Plasmamembranen enthielt. Die Cytosolfractionen führten zu einer 25%, die Membranfraktion zu einer 35% Erhöhung der Nervenzellpopulation.

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Curriculum Vitae

Andrea Sabine Maurer von Deutschland, geboren am 18.1.1957
Bietigheim/Württ.

- 1963 - 1967 Grundschule im Sand, Bietigheim
- 1967 - 1976 Gymnasium Bietigheim. Abschluss: Abitur in mathematisch -
naturwissenschaftlicher Richtung
- 1977 - 1979 Studium der Zoologie an der Southern Illinois University
at Carbondale, USA. Abschluss: Bachelor of Arts
- 1979 - 1984 Studium der Zoologie an der Universität Zürich
Abschluss: Diplom
- 1984 Ausführung der Dissertation unter der Leitung von
Prof.Dr.P.Tardent am Zoologischen Institut der
Universität Zürich
- 1984 - 1987 Anstellung als NF-Doktoandin

An der Universität besuchte ich bei folgenden Dozenten Vorlesungen und
Praktika:

Bachofen R., Billeter M., Birnstiel M., Burla H., Chen P.S., Christen
P., Cook C.D.K., Dübendorfer A., Hohl H., Honegger T., Humbel R.,
Jungen H., Jungen-Hauschteck E., Kägi J.H.R., Kubli E., Kummer H.,
Mindek G., Nöthinger R., Rast D., Ruffner H.P., Schmid V., Storrer H.,
Tardent P., Wanner H., Weissmann CH., Wehner R., Ziswiler V.

